Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 40. APRIL, 1939. NO. 4.

SECTION MEETINGS

IOWA	
Iowa State College	April 7, 1939
MINNESOTA	
University of Minnesota	March 15, 1939
MISSOURI	
St. Louis University	April 12, 1939
NEW YORK	
Rockefeller Institute	March 22, 1939
SOUTHERN CALIFORNIA	
Zoo Hospital, San Diego	March 25, 1939

10473

Metabolism of Pathogenic Bacteria Growing Under Aerobic Conditions in Carbohydrate-rich Culture Media.*

THEODORE E. FRIEDEMANN.†

From the Laboratory of Chemical Bacteriology, Department of Medicine, University of Chicago, Chicago.

The object of this paper is to show that the carbohydrate metabolism of *rapidly growing* bacteria in meat infusion-peptone culture medium, despite aerobic conditions, is almost entirely anaerobic. Since the culture medium contains most of the constituents of tissues

* This study was aided by the Bartlett Memorial Fund and the Douglas Smith Foundation for Medical Research, University of Chicago.

† With the assistance of Thaddeus C. Kmieciak.

TABLE I.
The results are expressed as mM or cc *N* per liter.

Flask	Cultural conditions	Glucose metabolized × 2 mM C_3	Lactic acid		Volatile acids mM	Undetermined non-volatile acidity cc *N*
			Yield mM	Yield %		
I	Anaerobic	32.4	28.1	87	4.5	1.4
II	Aerobic; 100 cc in Erlenmeyer flask	33.8	28.7	85	3.2	.8
III	Aerobic; thin layer in Kolle flask	33.0	27.7	84	3.7	.2

and since the metabolism is predominantly of carbohydrate, these experiments indicate the nature of the metabolic processes of rapidly growing bacteria in tissues. Representative data from 3 pathogens are given; these, as will be shown in a later paper, represent the products and the reactions most characteristic for pathogenic microorganisms as a group.

Freshly prepared, warm beef infusion to which had been added 1% of peptone, 0.7% of $Na_2HPO_4 . 12\ H_2O$, 1% of glucose and 2% by volume of serum, was inoculated with a virulent, rapidly growing, Type I pneumococcus. Measured volumes were transferred to sterile flasks as follows: I, 200 cc into a 200 cc volumetric flask, then immediately covered with oil; II, 100 cc into a 300 cc Erlenmeyer flask; III, 50 cc into a Kolle flask. The Kolle flask was immediately placed on its side and was not thereafter disturbed. Since the openings of flasks II and III and the volume of air above the medium were large, it was necessary to correct for the moisture loss by blanks.

Growth was rapid; it was about 90% complete in 8 hours. The cultures were acidified after about 24 hours of incubation at 37.5°C. They were then analyzed according to the procedures previously described.[1] The results, corrected for moisture loss, are shown in Table I.

Except for the small volume of dissolved oxygen initially present and traces of oxygen which may have entered subsequently through the oil, the condition in flask I may be considered anaerobic. Based upon the sugar consumed, the yield of lactic acid was 87%. This agrees with the results obtained by Hewitt.[2] Almost identical yields of lactic and volatile acids were obtained from flask II, the Erlenmeyer flask in which the conditions were aerobic. Again, from flask III, the Kolle flask which contained a thin layer of culture

[1] Friedemann, T. E., *J. Bact.*, 1938, **35**, 527.

[2] Hewitt, L. F., *Biochem. J.*, 1932, **26**, 464.

medium, the results agreed with those from the other flasks. The lactic acid yield was 84%, which was practically within the limit of error of the method.

Further evidence of the anaerobic character of the metabolism of pneumococci under relatively aerobic conditions can be seen in the results given in Table II in a previous paper.[1] In experiment 3, for example, 57.1 mM per 1 of glucose x 2 were metabolized and 25.3 mM of lactic acid‡ were produced. Other metabolic products were: 25.2 mM of formic acid, 12.2 mM of acetic acid, and 12.2 mM of ethyl alcohol. The increase of CO_2 was insignificant; less than 0.1 mM in 24 hours. The yield of formic and acetic acids and alcohol was approximately in the ratio of 2 to 1 to 1.§

This is in keeping with the theories of Virtanen, Karström, and Turpeinen,[3] Meyerhof and Kiessling,[4] and Neuberg and Kobel.[5] According to these theories, glucose, through triose, finally yields 1 mole each of glycerophosphate and phosphopyruvate. These are further metabolized as follows:

$$\text{Glycerophosphate} \rightarrow C_2H_5OH + HCOOH^{6}$$
$$\text{Phosphopyruvate} \rightarrow CH_3COOH + HCOOH^{7}$$

The calculated yield of products according to these anaerobic reactions was realized in this experiment. The small yield of CO_2 is further evidence of the anaerobic character of the metabolism.

The same maximum yield of approximately 2 moles of formic acid for each mole of acetic acid or alcohol can often be obtained from other bacteria when the growth is very rapid. The rapid acidification of the medium due to growth in the presence of an excess of sugar, appears to protect the formic acid from decomposition. Examples

‡ The yield of lactic acid, 44.3% in this experiment, illustrates the wide variations which may be obtained in the same culture medium under apparently the same conditions. The lactic acid yield is not always as constant as indicated by Hewitt.[2]

§ Lactic acid is obtained from actively growing pneumococci, even when the culture medium is exposed to air in rapidly moving thin layers. 50.0 cc of inoculated culture medium were introduced into a sterile 18 l bottle. The bottle was rapidly rotated during a period of 8 hours. The results, expressed as mM per liter, were as follows: sugar × 2 consumed, 22.3; lactic acid, 10.5; acetic acid, 11.1; CO_2, 11.0; undetermined non-volatile acidity, —1.1. *The absence of alcohol should be noted.* The lactic acid yield was 47%.

3 Virtanen, A. I., Karström, H., and Turpeinen, O., *Naturwissenschaften*, 1929, **17**, 877. More complete discussion is given in *Z. physiol. Chem.*, 1930, **187**, 7.

4 Meyerhof, O., and Kiessling, W., *Biochem. Z.*, 1933, **264**, 40; **267**, 313.

5 Neuberg, C., and Kobel, M., *Biochem. Z.*, 1934, **272**, 445.

6 Harden, A., *Proc. Chem. Soc.*, 1901, **17**, 57.

7 Neuberg, C., and Ringer, M., *Biochem. Z.*, 1915, **71**, 237.

TABLE II.

Growth 12 hours at 37.5°C in meat extract medium which contained 1% of Witte peptone, 1.8% of $Na_2HPO_4 \cdot 12H_2O$, and 1% of glucose. Results are expressed as mM or cc *N* acid per l.

Organism	Products						
	Glucose × 2 used mMC_3	Lactic acid mM	Formic acid mM	Acetic acid mM	Ethyl alcohol mM	Undetermined non-volatile acidity cc *N*	Lactic acid yield %
Eberthella typhosa	39.4	4.1	28.8	15.4	13.1	0.8	10.4
Escherichia coli	51.8	13.9	27.2	14.1	14.0	3.2	26.8

of such results|| are shown in Table II. The absence of succinic acid in this experiment, as indicated by the "undetermined non-volatile acidity," should be noted.

It is interesting to note that Grey and Young[8] obtained equal or higher yields of lactic acid from *Es. coli* in those flasks which contained oxygen above the surface of the medium. Growth, according to Grey, was most rapid in these flasks. Hewitt[2] found identical yields of lactic acid from pneumococci in tubes of culture media kept under anaerobic conditions and in similar tubes exposed to air.

These results are significant because they clearly indicate the nature of the metabolic processes of bacteria growing in tissues. Strictly anaerobic conditions are perhaps never encountered in normally functioning tissues. Such conditions may be approached in edematous traumatized areas with marked stasis or occlusion of blood vessels. It is no doubt realized in the purulent exudate in the alveoli during lobar pneumonia[9] and in the interior of abscesses. Most organisms, however, in the early stages of an infection, encounter relatively aerobic conditions. These are comparable to the limited aerobiasis obtaining in test tube or Erlenmeyer flask cultures. Our results from carbohydrate-rich culture media, under these relatively aerobic conditions are almost identical with those obtained under strictly anaerobic conditions. Despite the complexity of the culture medium, carbohydrate is the chief source of energy. The metabolic products are relatively few in number, and the volatile products may be obtained in the proportions indicated by the reactions shown above.

|| The yield of lactic acid in this experiment was lower and the yield of volatile products was higher than reported by previous investigators. All of the published data represent the activities of these 2 organisms after from one to 2 weeks of growth in a medium consisting only of peptone, dextrose, and $CaCO_3$.

[8] Grey, E. C., and Young, E. G., *Proc. Roy. Soc. London*, 1921, **92** B, 135.

[9] Friedemann, T. E., and Graeser, J. G., *J. Exp. Med.*, 1937, **67**, 481.

Tissues contain considerable quantities of metabolizable carbohydrate which consist of free sugar, glycogen and other polysaccharides. These sugars are readily metabolized by many pathogenic microorganisms (unpublished data) when added to culture medium or serum; the products in every instance have been the same as are obtained from glucose.

Summary and Conclusions. *Diplococcus pneumoniæ, Eberthella typhosa,* and *Escherichia coli* were grown 24 hours at 37.5°C in meat infusion or meat extract culture medium enriched with 1% of peptone, 0.7 to 1.8% of $Na_2HPO_4 . 12 H_2O$, and 1% of glucose. The pneumococcus was grown under anaerobic as well as highly aerobic conditions. The principal products were lactic acid, formic acid, acetic acid, and ethyl alcohol. The last 3 products appeared in the approximate ratio of 2 to 1 to 1. The reactions representing their formation most likely are: Glucose → hexose diphosphate → 2 triosephosphate → glycerophosphate and phosphoglycerate. Glycerophosphate → C_2H_5OH + HCOOH. Phosphoglycerate → phosphopyruvate → CH_3COOH + HCOOH. These are anaerobic mechanisms. Only a trace of CO_2 was obtained from pneumococci.

Rapidly growing bacteria may metabolize sugar almost entirely by anaerobic mechanisms when grown under relatively aerobic conditions. The same intermediary reactions are probably also utilized by microörganisms when growing in tissues in the course of an infection.

10474 P

Occurrence of Sporadic Bacillemia in Experimental Tuberculosis in Dogs.

JOHN S. HOWE. (Introduced by A. J. Nedzel.)

From the Department of Pathology, Bacteriology and Public Health, University of Illinois College of Medicine, Chicago, Ill.

Wilson[1] in summarizing the work on bacillemia in human tuberculosis up to 1933, concludes that bacillemia, detectable by present methods, is, except as a transitory phenomenon, rarely present until the disease has assumed an acute phase. Because of this discrepancy between the reported rare occurrence of bacillemia and the common demonstration of hematogenous tuberculous lesions at necropsy, it

1 Wilson, G. S., *Tuberculous Bacillemia,* London, 1933.

seems important to determine the frequency and significance of bacillemia as a transitory phenomenon in tuberculosis.

Corper,[2] using dogs as experimental animals, reports that large doses of tubercle bacilli given intravenously produced a rapidly fatal bacillemia without the formation of tubercles. Smaller doses of tubercle bacilli produced no bacillemia and no tuberculous lesions. A search of the literature reveals no report of daily blood-cultures over a period of several months, which would seem to be the most adequate means of determining the frequency of transitory or sporadic bacillemia.

In our experiment, dogs were used because of the relative ease of obtaining an adequate quantity of blood daily for culture, and because the relative resistance of the dog to tuberculosis is more closely parallel to that of the human, than is the low resistance of the rabbit and guinea pig.

Experimental tuberculosis was produced in dogs by the intravenous injection of large doses of a virulent human type of tubercle bacilli (H37) in a suspension of Kaolan, mineral oil, and normal saline. This method was found to produce embolic tubercles limited largely to the lungs and tending to heal or to progress slowly.

Tuberculous abscesses were produced by injection of a similar suspension of tubercle bacilli into the chest wall of dogs. These lesions tended to break down and discharge for a time and then heal.

Seventeen dogs in all were injected by the intravenous method and 3 into the chest wall, with doses of tubercle bacilli varying from 3 to 20 mg. Femoral arterial punctures were performed daily on all dogs, and from 3 to 5 cc of arterial blood withdrawn and injected directly into guinea pigs, either subcutaneously or intraäbdominally. Arterial blood was used because of the theoretical consideration stressed by Bock,[3] that the nearer the point of origin of the bacilli the blood is obtained, the greater the chance of demonstrating bacillemia. This is particularly true since the blood sample is obtained before it passes through the peripheral capillary bed. Direct inoculation of guinea pigs was used in preference to other culture methods, since it has been shown by Saenz[4] that guinea pig inoculation is both more sensitive and more reliable than cultural methods.

Endermal tuberculin-tests were done on all the guinea pigs before inoculation with the blood and at intervals afterward, up to 3 months after inoculation, when all were sacrificed and necropsied. The pres-

[2] Corper, H. J., and Vidal, C. B., *Am. Rev. Tuberculosis*, 1935, **32**, 575.

[3] Bock, H. E., *Klin. Wchnschr.*, 1936, **15**, 1138.

[4] Saenz, A., *Ann. Inst. Pasteur*, 1934, **52**, 645.

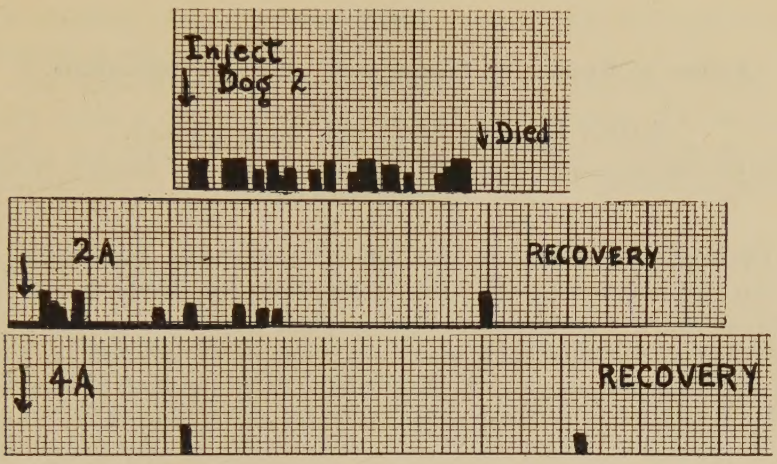


FIG. 1.

Showing the distribution of the sporadic bacillemia in a dog injected intravenously with a heavy dose of tubercle bacilli (2), one injected intravenously with a moderate dose (2A), and one injected subcutaneously with a moderate dose (4A). Each small square represents one day of observation, with the arrow indicating the time of injection. The heavy black squares indicate days on which bacillemia was demonstrated, with the height of the black square indicating roughly the degree of bacillemia.

ence of tuberculosis was established on the basis of the characteristic gross and microscopic pathology of injection-tuberculosis, supported by positive tuberculin-reactions and microscopic examination of smears for tubercle bacilli.

In the course of daily observations ranging from 1 to 3 months, 15 of the 17 dogs injected intravenously showed tubercle bacilli present in at least one blood sample. Three of these dogs dying of progressive tuberculosis showed the heaviest and most frequent periods of bacillemia, but 12 of the 14 dogs that recovered showed bacillemia on at least one day, and usually on several days at scattered intervals throughout the experiment.

Two of 3 dogs injected subcutaneously into the chest wall also showed sporadic bacillemia on at least one occasion.

The protocol of 2 typical dogs injected intravenously with a heavy and a moderate dose of bacilli and of a dog injected subcutaneously is appended.

These preliminary results indicate that even in an animal (dog) having a relatively high resistance to tuberculous infection, sporadic bacillemia occurs in a high percentage of animals infected experimentally, whether by the intravenous or subcutaneous route. This sporadic bacillemia depends quantitatively to some extent on the outcome of the infection, but is demonstrated even in a high percentage of animals that recover.

10475

Action of Ketene on Gonococcus and Meningococcus.*

ALDEN K. BOOR AND C. PHILLIP MILLER.

From the Department of Medicine and the A. B. Kuppenheimer Research Foundation, University of Chicago.

The toxicity inherent in the cells of gonococcus and meningococcus—commonly ascribed to "endotoxins"—probably handicaps them as effective antigens and restricts their usefulness as immunizing agents. We have made numerous attempts to diminish the toxicity of these organisms without impairing their antigenic properties.

Tamara and Boyd[1] reported detoxification of *B. dysenteriæ* Shiga by acetylation without alteration of its antigenic activity though the extent of this change is not apparent from the data presented in their preliminary communication. Acetylation was accomplished by means of ketene gas generated by the method of Herriott.[2] By the same technic Pappenheimer[3] was able to reduce very greatly the lethal action of diphtheria toxin without destroying its ability to combine and flocculate with antitoxin. Short exposure of toxic filtrates to ketene gas gave Goldie[4] similar results. Hyman[5] reports that ketene reduces the toxicity, but also the antigenicity of diphtheria toxin.

Ketene $\overset{H}{\underset{H}{}}>C=C=O$ is a gas which liquefies at −56°C. It reacts with amino acids, alcohols, amids and other compounds by acetylation.[3, 6, 7, 8]

Methods. Ketene was generated in an apparatus similar to that described by Herriott.[2] Vaporized acetone was passed over a hot platinum filament and then through a chamber cooled by solid carbon dioxide where polimers and unchanged acetone were removed by condensation. The ketene gas was bubbled slowly (about 1 bubble per second) through the bacterial suspension containing phenol red. Its reaction was maintained at pH 7.6-7.8 by frequent additions of 10% sodium hydroxide to neutralize the acetic acid as it formed.

* Aided by a grant from the National Research Council.

1 Tamura, J. T., and Boyd, M. J., *Science*, 1936, **83**, 61.

2 Herriott, Roger M., *J. Gen. Physiol.*, 1934, **18**, 69.

3 Pappenheimer, Alwin M., Jr., *J. Biol. Chem.*, 1938, **125**, 201.

4 Goldie, H., *Compt. Rend. Soc. Biol.*, 1937, **126**, 1, 4.

5 Hyman, L. W., *J. Bact.*, 1939, **37**, 228.

6 Staudinger, H., *Die Ketene*, Stuttgart, F. Euke, 1912.

7 Herriott, R. M., and Northrup, J. H. *J. Gen. Physiol.*, 1934, **18**, 35.

8 Stern, K. G., and White, A., *J. Biol. Chem.*, 1937, **122**, 371.

The duration of treatment with ketene varied in different experiments from 20 to 60 minutes.

The microörganisms were cultivated in pint medicine bottles on solid media consisting of tryptic digest of egg white, 1% dextrose buffer and agar. They were removed with saline, washed once and resuspended in distilled water, about 0.3 g moist, packed organisms in 5.0 cc suspension.

Cultures of the bacterial suspensions showed them to be sterile after 5 minutes' exposure to the gas. Control suspensions were, therefore, killed by heating at 70°C for 15 minutes. Acetic acid was added, then neutralized with sodium hydroxide in the same manner and quantity as the ketene-treated sample.

Toxicity of the bacterial suspensions was measured by their lethal action on white mice. Immediately after completion of the ketene treatment, series of 20 g mice were injected intraperitoneally with graded doses. Death usually occurred within 36 hours, but the mice were observed for 6 days. Preliminary experiments made clear the necessity of grading the dosage by small increments and of injecting enough mice with each dose to be statistically significant.

Results. Comparison of the mortality rates of the ketene-treated and control suspensions over the whole series of each titration showed that some degree of detoxification occurred in almost every one of a considerable number of experiments. At best it was never very great. An *average* result (neither maximal nor minimal) is exemplified in Table I.

Meningococcus behaved similarly to gonococcus.

Duration of detoxification. The chemical reaction responsible for detoxification is in whole or in part a reversible one. This fact is demonstrated by the experiment shown in Table II.

The dose of acetylated gonococci which killed only 2 of 6 mice was repeated after one week's storage in the ice box and it then killed all of 6 mice.

TABLE I.

Mortality of White Mice Injected Intraperitoneally with Acetylated (for 40 min.) or Control Suspensions of Gonococcus.

Dose, cc	Acetylated suspensions	Control suspension
1.4	5/8*	5/5
1.0	4/8	7/8
.75	2/8	4/8
.5	2/8	5/8
.3	0/8	3/8
.2	0/8	2/8

*Numerator denotes number of deaths; denominator denotes number of mice injected.

TABLE II.
Reversibility of Detoxification by Acetylation.

	Dose, cc	Acetylated* suspension	Control suspension (not acetylated)
Gonococcus suspension injected immediately after ketenization	0.5	2/6	6/6
Same gonococcus suspension injected 6 days after ketene treatment	0.5	6/6	

* Numerators indicate the number of mice killed by the injection. Denominators show the total number of mice injected in this experiment.

Effect of acetylation on the antigenic properties of gonococci. Three rabbits were immunized by a series of intravenous injections with suspensions of gonococci treated with ketene immediately before each injection. These rabbits survived weekly intravenous injections, increasing from 0.5 cc to 4.0 cc of the concentrations previously mentioned, without mortality. At the end of 6 weeks antiserum showed about the same antibody content by precipitin test and the same degree of specificity as that made by injections of unacetylated organisms or gonococcus protein.[9]

Conclusions. The toxicity of gonococcus and meningococcus cells is appreciably but temporarily reduced by acetylation with ketene.

10476 P

Experimental Intersexuality: Masculinization of Female Rats by Postpartum Treatment with Anterior-Pituitary-Like Hormone.*

R. R. GREENE AND M. W. BURRILL. (Introduced by A. C. Ivy.)

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

Papanicolaou and Falk[1] reported a masculinization of female guinea pigs due to treatment with anterior-pituitary-like hormone. This masculinization consisted of growth and enlargement of the clitoris. They also noted a "masculinizing effect" on the skeletal musculature due to the same treatment.[2] These "masculinizing" effects were associated with a marked development of the interstitial

[9] Boor, A. K., and Miller, C. P., *J. Exp. Med.*, 1934, **59**, 63.

* Supported in part by a grant from the Josiah Macy, Jr., Foundation.

[1] Papanicolaou, G., and Falk, E. A., PROC. SOC. EXP. BIOL. AND MED., 1934, **21**, 750.

[2] Papanicolaou, G., and Falk, E. A., *Science*, 1938, **87**, 238.

cells of the guinea pig ovary. Presumably the hypertrophied interstitial tissue produces an androgenic substance. Hill[3] demonstrated that ovaries transplanted to the ears of castrated male mice produced an androgenic substance. He believes that the ability of these grafts to produce androgenic substance is conditioned by the relatively low temperature to which they are exposed.[4] Hill mentioned a "luteinization of the transplants." Deanesly[5] has repeated this work in rats and believes that the androgenic output is directly related to the amount of thecal luteinization present in the grafted ovaries. Selye, Collip and Thomson[6] have demonstrated that when treatment of female rats with APL is started at 6 days of age, a marked thecal luteinization is found by the 16th day. If daily treatment is continued, granulosal luteinization is not found until the animal is 30 to 40 days old. However, from the data presented by these authors one gains the impression that the theca lutein cells produce estrogenic substance (persistent estrus smears in the experimental rats). They do not mention any change in the clitoris. This literature appears, on the surface, to be discordant and for this reason we have undertaken to repeat Papanicolaou's work, using the rat instead of the guinea pig and making histological studies of the changes occurring in the ovary as well as in the clitoris.

Sixteen newborn female rats were treated daily with APL† in increasing dosages varying from 50 to 800 R.U. The period of treatment varied from 6 to 50 days. The total dosage varied from 600 to 13,000 R.U. Ovaries of animals treated for 6, 10, 30, 36, and 50 days have been examined microscopically. The remaining animals were killed at the age of 6 to 9 months. In the treated animals, at 17-18 days, the clitoris was noticeably enlarged as compared with control littermates (5 animals) and normal stock animals. At 19-21 days, the clitoris was enlarged to such an extent that the organ was evertable. At autopsy, 5-8 months after cessation of the treatment, the clitorides were still noticeably larger than normal and were still evertable.

A preliminary microscopical study of the ovaries revealed little or no change in the animal killed after 6 days of treatment. In animals killed after 10 days of treatment, definite thecal luteinization has been found. After 30, 36 and 50 days of treatment, marked thecal

[3] Hill, R. T., *Endocrin.*, 1937, **21**, 495.

[4] Hill, R. T., *Endocrin.*, 1937, **21**, 633.

[5] Deanesly, Ruth, *Proc. Roy. Soc.* (Series B), 1938, **126**, 122.

[6] Selye, H., Collip, J. B., and Thomson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 780.

† The APL was furnished by Ayerst, McKenna and Harrison, Ltd.

luteinization has been found in some areas together with large bodies of lutein cells, some with a central cavity and with degenerating ova.

The 3 adult clitorides (7-8 mos.) which have been examined microscopically show structural modifications. A well developed *os priapi* with anterior process is present in each. This structure is not found in the normal adult female, although a homologue exists in the newborn female. Treatment with APL during the first few weeks of life has stimulated this homologue to develop into the typical male structure. There is also some stimulation of the glans epithelium in these animals in that papillae are more numerous than in the normal clitoris. In one animal differentiation of the papillae into spines has taken place. These spines are typical male structures. Some stimulation of the cavernous structures was also noted.

Both Deanesly and Hill have concluded that the androgen produced by ovaries grafted into males is not testosterone. Lamar[7] and the present authors[8] have shown that progesterone, in large amounts, is androgenic in the rat. Progesterone is presumably produced by lutein cells. It is not known whether, in the normal female rat, progesterone is produced by the luteinized granulosa or by the luteinized theca cells. At any rate, it is conceivable that the androgen produced by the ovaries of these experimental animals may be progesterone.

Summary. The administration of APL to infantile female rats causes masculinization of the clitoris consisting of gross enlargement and the development of an *os priapi*.

10477 P

Melanophore Hormone of the Pituitatry Gland and Metabolic Stimulation.*

R. S. TEAGUE. (Introduced by E. M. K. Geiling.)

From the Department of Pharmacology, University of Chicago.

Collip and coworkers have recently reported metabolic stimulation in rabbits, guinea pigs, and rats occurring within a few hours following injection of certain pituitary extracts.[1, 2, 3] The substance in

[7] Lamar, J. K., *Anat. Rec.*, 1937, **70**, Suppl. p. 45.

[8] Greene, R. R., Burrill, M. W., and Ivy, A. C., *Endocrin.*, 1939, **24**, 351.

* Aided by a grant from the Otho S. A. Sprague Memorial Institute.

[1] O'Donovan, D. K., and Collip, J. B., *West. J. Surg.*, 1937, **45**, 564.

[2] O'Donovan, D. K., and Collip, J. B., *Endocrin.*, 1939, **23**, 718.

[3] Billingsley, L. W., O'Donovan, D. K., and Collip, J. B., *Endocrin.*, 1939, **24**, 63.

the extracts producing this stimulation was called the "specific metabolic principle" of the pituitary. From the similarity in chemistry and occurrence in extracts, as well as from certain differences between these and other known pituitary hormones, it was suggested that the specific metabolic principle and the melanophore hormone might be identical. In view of the previous report of Zondek and Krohn[4] that "Intermedin" did not affect the metabolism of rabbits, it seemed worth while to study the subject further.

Accordingly, the effect on oxygen consumption of various melanophore hormone preparations was studied in rats. A modified Benedict spirometer was used to measure oxygen consumption. The rats were kept at room temperature but the metabolism was measured at 28°C. Comparable results were obtained with normal, thyroidectomized and hypophysectomized rats, and with rats recently fed as well as rats fasted 12-18 hours.

The usual procedure was to measure the basal rate in the morning, inject the extract after 2-3 hours, and measure the oxygen consumption over the following period of 8 hours without removing the rat from the machine. Using this technic, basal oxygen consumption for normal rats averaged 211 1/sq.m./day, for thyroidectomized

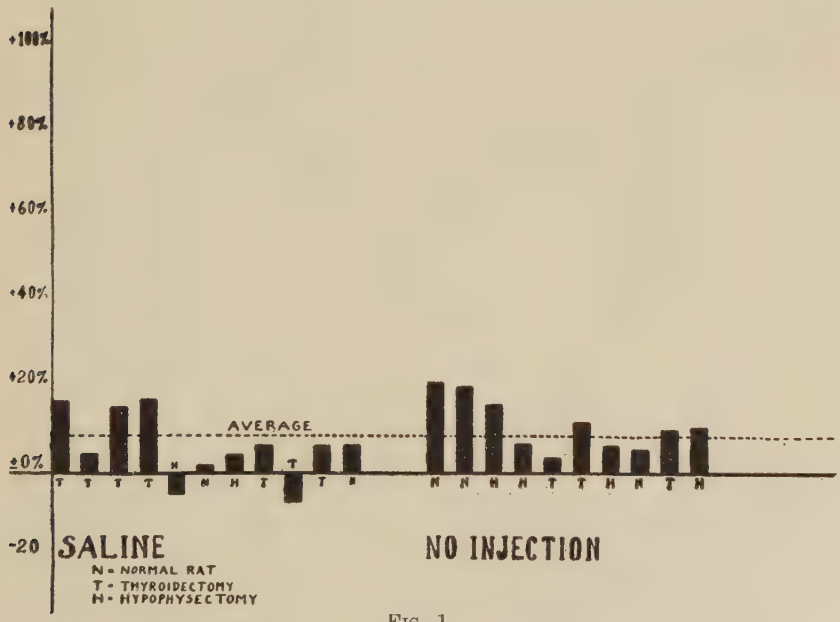


FIG. 1.

[4] Zondek, B., and Krohn, H., *Klin. Wchnschr.*, 1932, **11**, 1293.

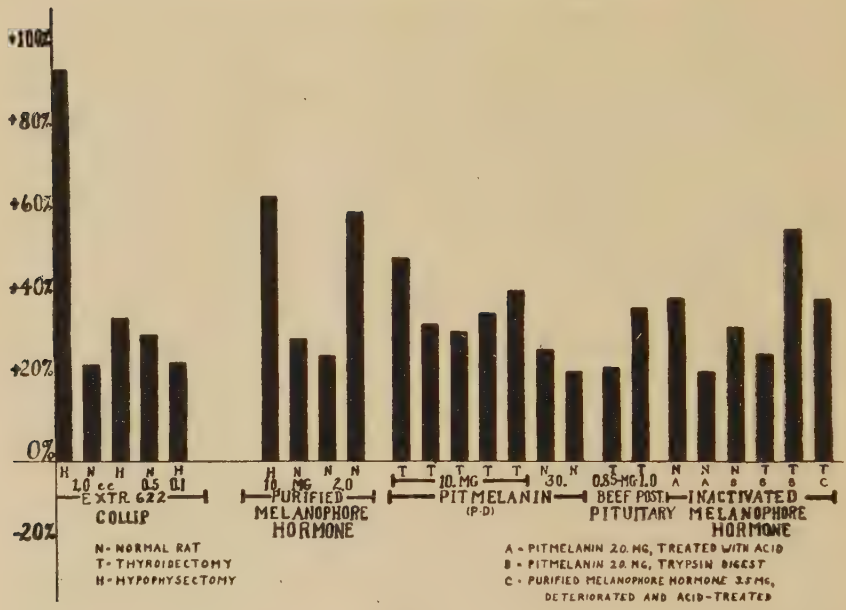


FIG. 2.

rats 160, and for hypophysectomized rats 137. Control studies are shown in Fig. 1. On the ordinate is the percentage change in oxygen consumption over the basal value. Each column represents the highest reading obtained after injection in one experiment. Fig. 2 summarizes only the positive findings with various pituitary extracts. The melanophore hormone content of each extract was determined by injection into hypophysectomized frogs which constitute a specific test object for the melanophore hormone.[5] No increase in oxygen consumption under 20% was considered significant. In 45 experiments with extracts rich in melanophore hormone a significant increase in oxygen consumption over controls was obtained in 18 experiments. No significant change was found in 27 experiments. It will be seen from Fig. 2 that a significant increase in oxygen consumption was obtained with a sample of Collip's extract 622† which is very rich in melanophore hormone. With this extract large doses, as a rule, gave a rise. With a purified melanophore hormone preparation prepared from beef posterior pituitary glands‡ and from

[5] Teague, R. S., Noojin, R. O., and Geiling, E. M. K., *J. Pharm. and Exp. Therap.*, 1939, **65**, 115.

† Obtained through the courtesy of Dr. J. B. Collip.

‡ Supplied by the Wilson Laboratories.

whale anterior lobe,§ by a method developed in this laboratory,[6] there were 4 positive to 9 negative experiments. With "Pitmelanin",‖ a partially purified melanophore hormone preparation, there were 2 positive to 7 negative experiments. Five significant and 5 non-significant results were also obtained with Pitmelanin after alkali treatment to destroy the slight contamination with the pressor principle (checked by cat assay). U.S.P. posterior pituitary powder contains a moderate amount of melanophore hormone. On one occasion a significant rise was obtained with this, while 3 other experiments were negative. After alkali treatment to destroy the pressor principle, an increase was obtained once, no change once.

The melanophore hormone in a sample of Pitmelanin was destroyed by boiling in concentrated HCl. Twenty mg of Pitmelanin so treated, containing only a trace of melanophore hormone, produced a significant increase on 2 occasions. After destruction of melanophore hormone by trypsin digestion, a similar sample of Pitmelanin still produced a significant increase in 4 experiments, no increase in 2. However, after inactivation of the melanophore hormone by repeated exposure to ultraviolet light, no significant metabolic stimulation was obtained in any of 8 trials. Likewise no results were obtained in 2 experiments using Pitmelanin inactivated by H_2O_2. Further control experiments with beef muscle, liver and kidney extracts in acetic acid have been negative.

It may be that some uncontrolled biologic variation in the rats may account for the variation in results. There may be a prosthetic group of a complex molecule of melanophore hormone affecting frog melanophores, while another prosthetic group of the same molecule stimulates mammalian metabolism. With such a hypothesis the results of melanophore destruction experiments may be explained by assuming that the prosthetic group stimulating metabolism is not affected by acid boiling or trypsin digestion, while that affecting melanophores is altered, while ultraviolet light or oxidation may destroy both. Another explanation is that metabolic stimulation is produced by a breakdown product of the melanophore hormone. Finally, the possibility of a non-specific effect on metabolism with such concentrated extracts has not been entirely eliminiated. As with so many other problems in endocrinology, the isolation of the chemically pure hormone may be required to settle the problem.

[6] Fostvedt, G., personal communication.

§ Obtained as a result of a grant from the Rockefeller Foundation to Dr. E. M. K. Geiling.

‖ Supplied by Dr. O. Kamm of the Research and Biological Laboratories of Parke, Davis and Co.

10478 P

Hyperthyroid Splanchnomegaly After Hypophysectomy.

H. G. Swann.

From the Department of Physiology, University of Chicago.

Experimental hyperthyroidism causes a considerable splanchnomegaly,[1] the enlargement including heart, liver, spleen, kidneys, and adrenals. The same can be produced by the injection of anterior pituitary extracts.[2] Moreover, the converse condition, splanchnomicria, occurs after either thyroidectomy or hypophysectomy.[3] It is therefore evident that both these glands influence the size of the splanchnic viscera. The same generality holds for somatic growth: in the absence of either of these glands it is deficient and experimental gigantism can be produced with pituitary extracts. Somatic growth appears to be controlled primarily by the hypophysis, however; the lack of growth in the cretin is usually ascribed to defective hypophyseal growth-promoting hormone secretion which follows the thyroidectomy.[4, 5] Good evidence in favor of this hypothesis is that administered thyroid will not produce somatic growth in the hypophysectomized rat.[5]

In order to determine whether splanchnic visceral growth is controlled by the same sort of mechanisms as somatic growth, hypophysectomized rats were given thyroxin for a period of a month after operation. Normal controls were also injected. The size of the viscera of untreated rats served as standards, the normal size for a given weight being determined in a large series of rats and expressed as 100%. Similar standards were set up for hypophysectomized rats. In Table I the effect of various doses of thyroxin, administered for a month, on the size of some of the splanchnic viscera is shown.

---

1 Hoskins, E. R., *J. Exp. Zool.*, 1916, **21**, 295; Herring, P. T., *Quart. J. Exp. Physiol.*, 1917, **11**, 230; Cameron, A. T., and Carmichael, J., *J. Biol. Chem.*, 1920, **45**, 69.

2 Putnam, G. J., Benedict, E. B., and Teel, E. M., *Arch. Surg.*, 1929, **18**, 1708; Murphy, R., Lowther, S., and Pagniello, L., 1938, **124**, 110.

3 Smith, P. E., *Anat. Rec.*, 1930, **45**, 205; White, W. E., *Proc. Roy. Soc.*, B, 1933, **114**, 64; Perla, D., *J. Exp. Med.*, 1936, **63**, 599.

4 Flower, C. F., and Evans, H. M., *Anat. Rec.*, 1925, **29**, 383; Zeckwer, I. T., Davison, L. W., Keller, T. B., and Livingood, C. S., *Am. J. Med. Sci.*, 1935, **190**, 145; but see Smith, P. E., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 1252; Salmon, T. N., *Endocrin.*, 1938, **23**, 446.

5 Smith, P. E., Greenwood, C. F., and Foster, G. L., *Am. J. Path.*, 1927, **3**, 669.

TABLE I.
Effect of Hypophysectomy on Hyperthyroid Splanchnomegaly.

Group No.	Preparation	No. of rats	Dose of Thyroxin, mg/kg given thrice weekly	Individual avg wt, expressed as % of normal				
				Liver	Kidneys	Spleen	Heart	Adrenals
1	Normal	55	0	100	100	100	100	100
2	"	7	0.5	96	114	216	105	237
3	"	8	1.0	135	137	365	118	317
% change in weight of group 3 over normal (group 1)				+35	+37	+265	+18	+217
4	Hypophysectomized	22	0	72	77	93	87	55
5	"	7	0.17	100	122	240	108	41
6	"	4	0.5	122	148	256	131	43
7	"	6	0.75	100	125	217	110	55
8	"	3	1.0	105	118	219	121	50
Avg of groups 5, 6, 7, 8				107	128	233	118	47
% change in wt of above average over hypophysectomized (group 4)				+49	+65	+150	+36	—15

The size of the viscera of untreated hypophysectomized rats underwent considerable reduction (Group 4). But treatment of hypophysectomized rats with thyroxin caused a splanchnomegaly in all organs except the adrenals. If the organ weights are averaged and then compared with those of untreated hypophysectomized rats (last line of Table I), the degree of splanchnomegaly is found to be as great or even greater than that in unoperated hyperthyroid rats. Evidently the thyroid-pituitary mechanisms controlling visceral growth are not the same as those controlling somatic growth. In the latter, the thyroid partially controls the pituitary which then stimulates somatic growth; this same mechanism is here demonstrated to apply for the adrenals. But in the case of the other splanchnic viscera, growth can occur with the pituitary absent; hyperthyroidism causes growth directly, and hyperpituitarism indirectly by its thyrotropic hormone.

The splanchnomegalogenic doses of thyroxin for the hypophysectomized rat were found to be smaller than those for the normal rat, in confirmation of the observation[5] that hypophysectomized rats are more sensitive to thyroxin than normals. Indeed, 3 mg of thyroxin per kilo weekly were found lethal to almost all hypophysectomized rats.

Summary. The hypertrophy of the liver, kidneys, heart, and spleen caused by hyperthyroidism occurs in the absence of the pituitary; that of the adrenals does not.

10479

Motility During Sleep in Psychopathic and Mentally Defective Subjects.*

F. J. Mullin, N. Kleitman and S. Titelbaum.

From the Department of Physiology, University of Chicago, and University of Texas Medical Branch.

Extensive studies on the sleep of the insane by Ladame[1] and Courbon[2] have attempted to correlate particular types of insanity with various types of sleep habits. Muncie[3] and Richter[4] have also made observations on the sleep characteristics of psychotic patients. Forbes[5] and Page[6] have compared the motility during sleep of psychopathic patients with that of normal individuals. Their findings indicate that in several types of psychopathic patients the motility during sleep is fairly comparable to that of normal individuals. The motility of catatonics in Page's study was found to be quite low and more or less uniform throughout the night, while normal individuals and manic-depressives and postencephalitic parkensonians all moved more during successive thirds of the night.

Using recording devices[7] for measuring the time spent in motility, the amount of motility, and the distribution of the night's movements, supplemented by regular hourly observations recorded on sleep charts by nurses and attendants, we studied a number of psychopathic patients and mentally defective individuals.

Psychopathic patients in the Division of Psychiatry of the University of Chicago Clinics (Billings Hospital) whom we studied were divided into 3 groups: psychoneurotics, schizophrenics, and affective psychotics. Only those subjects whose sleep motility was

* This work was aided by a grant from the Rockefeller Foundation. We wish to thank the various physicians and nurses and attendants who so generously gave their time and effort in cooperating with us in obtaining the records used in this study. We also wish to express our appreciation for the privilege of studying these subjects to Dr. D. Slight of the University of Chicago Clinics, Dr. L. R. Brown of the State Psychopathic Hospital at Galveston, Texas, and Dr. F. A. Causey of the Lincoln State School and Colony, Lincoln, Illinois.

1 Ladame, C., *Schweiz. Arch. f. Neurol. u. Psychiatr.*, 1923; **13**, 371.

2 Courbon, P., *Rev. Neurol.*, 1927, **1**, 869.

3 Muncie, W., *Bull. Johns Hopkins Hosp.*, 1934, **55**, 131.

4 Richter, C. P., *Arch. Neurol. Psychiat.*, 1934, **31**, 149.

5 Forbes, W. T., *Psychiat. Quart.*, 1934, **8**, 538.

6 Page, J. D., *Arch. Psychol.*, 1935-36, **28**, 1.

7 Kleitman, N., Cooperman, N. R., and Mullin, F. J., *Am. J. Physiol.*, 1933, **105**, 574.

observed for at least 10 days were used in the analysis of the results. The results of 408 nights' sleep of 24 patients are given in Table I. Thus we see that all of these psychopathic patients showed sleep habits and motility quite similar to that found in normal persons.[8] Just as in the normal individual, the incidence of spontaneous awakening in each class of psychopaths was over twice as great in the second half of the night as during the first half of the night's sleep. There was also considerable individual variation among these subjects, though the time spent in motility for the group as a whole was rather less than the average time spent moving about by normal persons.

TABLE I.

Classification	No. of patients	No. of nights	Motility per half night—sec/hour 1st half	Motility per half night—sec/hour 2nd half	Time taken to fall asleep, in min.	Avg No. of spontaneous interruptions per night	Avg duration of interruptions, in min.
Psychoneurotics	10	226	14.4	20.9	23	0.6	27
Schizophrenics	8	95	16.4	22.8	45	1.0	39
Affective Psychotics	6	87	18.9	21.5	36	0.3	19

At the State Psychopathic Hospital in Galveston, Texas, studies were conducted using a simple work-adder device for recording the extent of motility during the night. Four psychoneurotic patients and 8 patients showing various mild degrees of depression were studied for a total of 437 nightly records. Most of these patients showed a rather even distribution of motility throughout the night, similar to that observed in schizophrenics by Page. There was considerable individual variation among these patients in Galveston, although except for the first hour the average motility of the group was slightly lower than that found in normal individuals. A few of these patients tended to be quite restless after getting in bed and before going to sleep. No direct correlation could be made between the motility of the sleeping subjects and the room temperatures.

Terman and Hocking[9] studied the sleep in normal children of various ages and also in mental defectives of various mental age levels. They found that normal children of the lower age group slept more than the mental defectives of the same mental age level, while at higher age levels the normal children and the mentally de-

[8] Kleitman, N., Mullin, F. J., Cooperman, N. R., Titelbaum, S., *Sleep Characteristics*, 1937, Univ. of Chicago Press.

[9] Terman, L. M., and Hocking, A., *J. Educ. Psychol.*, 1913, **4**, 138, 199, 269.

fective subjects slept about equally. We studied 36 mentally defective individuals for a total of 531 nights. The subjects were divided into 3 classes: 10 idiots (average I.Q. of 18), 12 imbeciles (average I.Q. of 34), and 14 high grade mental defectives (average I.Q. of 58). These mentally defective subjects showed considerable individual variation and there was only a slight tendency for a greater amount of movement in the second half of the night in the imbeciles and high grade mental defectives, while the idiots had a slight tendency for the majority of the motility to occur in the first part of the night. On the whole, the motility in these subjects was more evenly distributed throughout the night than is the case for normal individuals, but there was no marked difference in the sleep of these mentally defective individuals and that of normal persons. No correlation was found to exist between the type of motility shown by these patients and their chronological ages.

Seven children, whose I.Q.s were all above 60, at a private school for the mentally and physically subnormal were also studied. Although 2 of the children did not show a typical distribution of motility, all the others definitely moved more in the later part of the night. The group motility curve for all 7 was quite similar to that of normal children.

Thus we see that the sleep of certain types of psychopathic patients or of mentally defective individuals may be quite similar to that of normal persons, as judged by their night motility, but that normal people seem to have a greater amount of movement during the night and it is more unevenly distributed in favor of the second half of the night. Certainly, we could make no correlation with mental condition in any individual case on the basis of a study of sleep motility, both because of wide individual variations found and because of the tendency of all of these subjects to have similar sleep habits and characteristics to those of normal people.

10480

Reduced Glutathione of Tissues and Insulin Sensitivity.

R. Levine, O. Hechter, A. Grossman and Samuel Soskin.

From the Department of Metabolism and Endocrinology, Michael Reese Hospital, and the Department of Physiology, University of Chicago.*

The sensitivity of an animal to insulin is usually judged by the extent and duration of the depression of the blood sugar level after the administration of insulin. The factors which are usually considered to exert an important influence upon the insulin depression curve are: the available carbohydrate stores in the liver and the ease with which these stores may be mobilized.[1] The state of the endocrine glands is important in both these respects, and it is well known that adrenalectomy,[2] hypophysectomy,[3] and thyroidectomy[4] increase the sensitivity to insulin. Another important factor in insulin sensitivity, to which less attention has been paid, is the rate of destruction or inactivation of insulin by the tissues of the body. That this factor is significant is shown by the well-established fact that the same amount of insulin becomes more effective when administered in divided doses or by prolonged constant injection than when given in a single dose.[5]

The exact mode of insulin inactivation in the body is unknown, but ever since it was shown that insulin is a protein, the supposition has been that it is destroyed by proteolytic enzyme systems.[6] However, it is also known that the physiological action of insulin depends upon the integrity of its S-S groups,[7, 8] and that the *in vitro* reduction of these groups by sulphydryl compounds renders the insulin inert upon subsequent injection.[9–13] That a similar mode of inacti-

* Aided by the Max Pam Fund for Metabolic Research.

1 Zucker, T. F., and Berg, B. N., *Am. J. Physiol.*, 1937, **119**, 539.

2 Long, C. N. H., and Lukens, F. D. W., *J. Exp. Med.*, 1936, **63**, 465.

3 Houssay, B. A., *New Eng. J. Med.*, 1936, **214**, 961.

4 Bodansky, A., Proc. Soc. Exp. Biol. and Med., 1924, **21**, 46.

5 Scott, E. L., and Dotti, L. B., *J. A. M. A.*, 1932, **50**, 511.

6 Jensen, H. F., *Insulin*, Oxford University Press, London, 1938.

7 Stern, K. J., and White, A., *J. Biol. Chem.*, 1937, **117**, 95.

8 Stern, K. J., and White, A., *J. Biol. Chem.*, 1937, **119**, 215.

9 DuVigneaud, V., Fitch, A., Pekarek, E., and Lockwood, W. W., *J. Biol. Chem.*, 1931, **94**, 233.

10 Freudenberg, K., and Eyer, H., *Z. f. physiol. Chem.*, 1932, **213**, 226.

11 Wintersteiner, O., *J. Biol. Chem.*, 1933, **102**, 473.

12 Freudenberg, K., and Wegmann, T., *Z. f. physiol. Chem.*, 1935, **233**, 159.

13 Kather, E., *Arch. f. exp. Path. u. Pharmak.*, 1937, **185**, 323.

vation applies to insulin in the living organism is indicated by the work of Jacobs, who has recently demonstrated that the administration of cysteine decreases the reaction to subsequently injected insulin.[14]

These experimental considerations raise the question as to what rôle the normally occurring sulphydryl compounds of the tissues play in insulin inactivation, and therefore in the sensitivity to insulin. Such compounds are glutathione, which is present in the body almost wholly in its reduced form (GSH), and the sulphydryl groups of the tissue proteins. In the absence of satisfactory methods for determining the latter we have tried to obtain a partial answer to the above question by determining the GSH content of blood, muscle and liver in normal animals, and in animals known to be hypersensitive to insulin.

Methods. The GSH of fixed tissues was determined in rats. For practical reasons the estimations of blood GSH were made on dogs and also on humans. The GSH was estimated by the iodometric titration of 4% trichloracetic acid filtrates of blood and tissues. Vitamin C was determined by titration with 2-6 dichlorphenol-indophenol and the equivalents subtracted from the iodometric titration.[15] The results obtained for normal animals agreed quite well with figures obtained by the specific manometric method of Woodward[16] and the cadmium lactate method of Binet and Weller.[17] All determinations were run in triplicate.

The completeness of the hypophysectomies as well as of the adrenalectomies was checked at the time the animals were sacrificed. As a contrast to hypophysectomy we administered to normal rats an extract of the anterior pituitary gland† which we have elsewhere shown is able to maintain the blood sugar level of fasting hypophysectomized dogs. Our rats received 1 cc of "Phyone" subcutaneously per day (divided into 3 doses), for 2 days prior to the day on which they were sacrificed for tissue analyses.

Results. The chief results, namely the GSH content of liver and muscle in rats, are summarized in Table I. The results on the GSH of blood, and of some additional exploratory experiments, appear in the text below.

---

[14] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 305.

[15] Fujita, A., and Iwataka, D., *Bioch. Z.*, 1935, **277**, 284.

[16] Woodward, G. E., *J. Biol. Chem.*, 1935, **109**, 1.

[17] Binet, L., and Weller, G., *Bull. Soc. Chim. Biol.*, 1936, **18**, 358.

† "Phyone" (Wilson and Co.) for supplies of which we are indebted to Dr. David Klein.

TABLE I.

Condition	No. of rats	Liver GSH mg %	% change	Muscle GSH mg %	% change
Normal	18	217 ± 26	0	32 ± 5	0
Hypophysectomized	12	134 ± 29	—38	38 ± 5	+18
Adrenalectomized	8	137 ± 24	—37	37 ± 3	+16
Normals given Phyone	8	243 ± 21	+12	27 ± 3	—16

GSH in Liver and Muscles. It is apparent from Table I that the GSH of the liver of hypophysectomized and adrenalectomized rats is significantly lower than that of normal rats. A smaller number of determinations on livers of normal and hypophysectomized dogs yielded similar results. The average liver GSH for 3 hypophysectomized dogs was 153 mg % as compared with an average of 204 mg % for the livers of 4 normal dogs. Normal rats given "Phyone" showed an increase in the liver GSH. The increase is small as compared to the decrease after hypophysectomy, but the factors of dosage and of the preëxisting level of anterior pituitary hormone in the tissues must be considered in evaluating the effects of the administered hormone.

Table I shows variations of doubtful significance between the GSH of the skeletal muscles, as compared to the definite changes in liver GSH. However, the changes in the muscle, if significant, are consistently opposite to those in the liver. We are unable to explain this difference between liver and muscle at the present time. One possibility is suggested in our discussion below.

GSH in Blood. The essential finding as regards blood was that it did not parallel the GSH content of the fixed tissues either in normal animals and humans, or in hypophysectomized dogs, or in humans suffering from diabetes mellitus, acromegaly, hepatitis and other clinical conditions. In both man and dog under all the above conditions the GSH of blood ranged from 25-40 mg %, depending upon the red blood cell volume, as determined by hematocrit readings, in the particular individual. The latter relationship may explain the variation in blood GSH observed by others[18, 19] but not found by us.

We attempted to lower tissue GSH artificially by the administration of iodoacetic acid and of alloxan, both of which substances have been previously shown to oxidize GSH *in vitro*.[20, 21] It was

[18] Zunz, E., and Vesselovsky, O., *Ann. de physiol.*, 1937, **13**, 1064.

[19] Houssay, B. A., *New Eng. J. Med.*, 1936, **214**, 1002.

[20] Goddard, E., and Schubert, M. P., *Bioch. J.*, 1935, **29**, 1009.

[21] Lieben, F., and Edel, E., *Bioch. Z.*, 1933, **259**, 8.

our purpose to determine whether such lowering of the tissue GSH would cause an increased sensitivity to insulin. Our attempts were not successful. The iodoacetic acid which lowered the tissue GSH markedly (from 217 to 113 mg %), also caused coincident hyperglycemia due to liver glycogenolysis, which prevented determinations of insulin sensitivity. The alloxan failed to lower tissue GSH *in vivo*.

Discussion and Summary. Our results show that the GSH of liver is significantly lower than normal in animals known to be hypersensitive to insulin. This relationship was not observed for skeletal muscle or blood. This may indicate that the liver is more important than other tissues for insulin inactivation. However, as noted in our introductory remarks, the GSH is only one of the factors which may be involved in this type of inactivation. It is possible that the –SH groups associated with the tissue proteins may play an important rôle in determining the sensitivity to insulin, and that this factor may be relatively more important in muscle than in liver. This possibility is indicated by the preliminary note of Lehmann[22] which appeared while our work was in progress. He stated that insulin is inactivated *in vitro* by extracts of rabbit muscle and that two substances in the extract are responsible: (1) a dialyzable, thermostable factor, probably GSH, and (2) a non-dialyzable, thermolabile factor, probably proteins containing –SH groups.

It is apparent that final conclusions as to the relationship between the sulphydryl compounds normally present in the tissues, and the inactivation of insulin, and hence insulin sensitivity, must await further work in which all the –SH compounds are quantitatively determined and compared with insulin sensitivity. However, this relationship may be tentatively assumed on the basis of the following summary:

(1) Insulin is inactivated *in vitro* by sulphydryl compounds.[9-13]

(2) Muscle extracts inactivate insulin *in vitro* by virtue of two factors which are most probably GSH, and proteins with sulphydryl groups.[22]

(3) Cysteine injected *in vivo* results in a decreased sensitivity to insulin.[14]

(4) The GSH content of the livers of animals known to be hypersensitive to insulin is significantly lower than that of normal animals.

[22] Lehmann, H., and Schlossmann, H., *J. Physiol.*, 1938, **94**, 15P.

10481 P

Sulfapyridine and Serum Therapy in Experimental Lobar Pneumonia of Rats.

MAXWELL KEPL AND F. D. GUNN.

From the Department of Pathology, Northwestern University Medical School.

This group of experiments was planned to test the effect of sulfapyridine ("M. and B. 693", "Dagenan"*) upon the course and outcome of experimental pneumonia in rats. The report by Whitby[1] and several reports by American workers have indicated that this new compound is superior to sulfanilamide in combatting pneumococcal infections. The experiments so far reported have been confined mainly to infections of mice and rats by intraperitoneal and subcutaneous injection of various types of pneumococci. Cooper, Gross and Lewis[2] have recently reported upon Type II pneumococcal meningitis in rats.

In our first group of experiments we have used only one strain of pneumococcus, a Type I strain with high virulence for rats which we obtained from Dr. O. H. Robertson. It has been used for several years in producing experimental pneumonia. Young adult rats weighing 160 to 260 g were used for the experiments. The method used for production of the pulmonary infections has been previously described.[3, 4] Eighteen-hour dextrose-bouillon cultures were diluted with physiological saline, the final dilution in 5% commercial gastric mucin, and each animal received 0.1 cc of viscid mixture by intrabronchial insufflation. The sulfapyridine was suspended in 15% gum tragacanth or, more conveniently, in 5% mucin and administered by stomach tube, each animal receiving about 2 cc of mixture. Type I therapeutic serum (Felton)† was injected intraperitoneally diluted to 2 cc with saline.

In a preliminary orientation experiment, 18 rats were infected with cultures diluted to 10^{-4} and 12 of them treated with the sulfapyridine four hours later. Each of 6 animals received 125 mg for the first dose, followed by 60 mg at daily intervals for 5 days. Six

* "Dagenan" kindly furnished by Merck and Company, Inc., Rahway, N. J.

1 Whitby, L. E. H., *Lancet*, 1938, **1**, 1210.

2 Cooper, F. B., Gross, P., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 37.

3 Nungester, W. J., and Jourdonais, L. F., *J. Bact.*, 1935, **29**, 34.

4 Gunn, F. D., and Nungester, W. J., *Arch. Path.*, 1936, **21**, 813.

† Donated to us by Parke, Davis and Company, Detroit, Mich., and by E. R. Squibb and Sons, New York.

received 60 mg as the initial dose and 36 mg at daily intervals for 5 days. The 6 untreated controls died with lobar pneumonia and fibrino-purulent pleuritis within 48 hours. All 6 of those receiving the smaller dose of the drug were dead within 48 hours. Three of those receiving the larger dose were dead by the seventh day and the 3 surviving until the twenty-fourth day apparently in good condition were sacrificed and examined. Two showed only the India ink tracer in the left lung. The other had soft fibrous adhesions and encapsulated empyema of the left side.

In the second experiment, the same infecting dose was used in 30 animals. Of the 6 untreated controls, 5 were dead within 48 hours and the sixth was dead on the fifth day after injection. All showed pneumonia of the left lung. Of 6 animals receiving an initial dose of 60 mg of sulfapyridine, followed by 5 daily doses of 36 mg, 2 survived indefinitely. Of the 6 receiving 125 mg as an initial dose and 60 mg maintenance dose, 3 died as the result of infection and one was accidentally killed in attempting to pass a stomach tube (perforation of esophagus). Of 6 animals receiving Type I anti-pneumococcic serum in doses of 200 units each, 4 hours after infection and 100 units on each of the 5 succeeding days, 3 died. Finally, 6 animals were given both serum and sulfapyridine, the

TABLE I.
Efficacy of Sulfapyridine and Specific Serum in Type I Pneumonia.
Group 1. Treated 4 hours after infection.

No. rats	Avg wt g	Culture dilution	Therapy	Total dose	Survivals		Deaths	
					No.	%	Av. surviv.	Traumatic
24	190	10^{-4}	None		0	0	2.1	
12	180	"	Sulfapyr.	240 mg	2	17	3.7	
6	180	"	"	425	3	50+	4.	1*
18	230	"	"	850	10	55+	4.5	3*
6	190	"	Serum	700 U	3	50	5.3	
6	190	"	Sulfapyr. + serum	425 mg 700 U	5	83+	3.	
		Group 2.	Treated 18 hours after infection.					
22	175	10^{-4}	None		0	0	1.6	
12	180	"	Sulfapyr.	425 mg	3	25	1.6	
11	180	"	"	850	3	27+	5.7	
10	180	"	Serum	1400 U	5	50	1.8	
12	200	"	Sulfapyr. + serum	850 mg 1400 U	1	8+	4.5	3*
11	190	10^{-5}	None		0	0	2.5	
12	190	"	Sulfapyr.	1250 mg	2	16+	2.2	2*
12	170	"	Serum	1250 U	3	25	1.7	
12	180	"	Sulfapyr. + serum	1250 mg 1250 U	4	33+	2.7	

* In computing percentage of survivals, traumatic deaths were included with deaths from infection but were not included in the average survival period of rats dying spontaneously. This method works to the disadvantage of groups receiving sulfapyridine.

latter in doses of 60 mg as initial dose and 36 mg daily for maintenance dose. Only one of these died and the necropsy revealed a minimal lesion in the left lung, insufficient to account for the fatal outcome. The others survived indefinitely. The results of these 2 experiments are combined as Group 1 in the Table I, all animals in the group receiving the same infecting dose.

In a third experiment, therapy was not begun until 18 hours after infection. Sixty-seven rats were infected with 0.1 cc of a 10^{-4} dilution of an 18-hour culture and 47 rats with a 10^{-5} dilution. Of the former, 12 animals received a total of 425 mg of sulfapyridine over a period of 6 days. The initial dose in every case was approximately twice the maintenance dose. Three of these survived. Of 11 rats in which the total quantity of drug administered was 850 mg, 3 survived indefinitely. While the mortality was about the same in the group receiving the larger dose as in that receiving 425 mg, the average period of survival in those dying of infection was longer in the former (5.7 days in rats receiving 850 mg and 1.6 days in those receiving 425 mg). Ten rats in this series were treated by intraperitoneal injection of 400 U. of Type I antipneumococcic serum on the day after infection, followed by 200 U. daily for 5 days. Five of these survived indefinitely and 5 died within 1 to 4 days (avg 1.8 days). Twelve animals were given both sulfapyridine (850 mg) and serum (1400 U.). The results are shown in Group 2 of the table. One animal survived, 3 died as the result of trauma and the survival time of those dying was prolonged (4.5 days). Of 22 untreated controls which received the same infecting dose of pneumococci, all died in 1 to 4 days (avg 1.6 days) after infection.

In a group of 47 rats the infecting dose was smaller (dilution 10^{-5}) than in the preceding experiments. Twelve received 250 mg of sulfapyridine daily for 5 days, a total of 1250 mg for each rat. Two survived, 2 died of trauma and the average survival time of those dying of infection was 2.2 days. Twelve were given 250 U. of serum on each of 5 days; 3 survived and the average survival time of those dying was 1.7 days. Of 12 rats receiving both serum and drug, 4 were alive at the end of 3 weeks and the average time of survival of the rest was 2.7 days.

Summary. A combination of type-specific serum, administered intraperitoneally, and sulfapyridine, administered by stomach tube, was more efficacious in the treatment of experimental lobar pneumonia of rats than either alone, when treatment was begun within 4 hours after infection. The optimal dose of sulfapyridine under the conditions of our experiments was 0.5 to 1 g per kilo of rat per

day. After the infection was well established, following intrabronchial insufflation of relatively large doses (0.1 cc of 10^{-4} and 10^{-5} of 18-hour bouillon cultures) of pneumococci, serum was more efficacious than sulfapyridine in preserving life, but a combination of the 2 did not show a significant reduction in mortality even with the smaller infecting dose.

10482

Factors Affecting the Vitamin B_1 Content of Evaporated Milk.

F. W. SCHLUTZ AND ELIZABETH M. KNOTT.

From the Department of Pediatrics, University of Chicago.

The work of several investigators who have studied the vitamin B_1 content of evaporated milk has been recently reviewed by Daniels.[1] She reported two trends in results: certain workers had found about 50% destruction of vitamin B_1 when evaporated milk was compared with raw milk, whereas others had observed only 0-20% destruction. Using a modification of the 10-day rat growth technic of Schlutz and Knott,[2] Daniels assayed raw Guernsey milk and commercial evaporated milk. Her results indicated 60% less vitamin B_1 in the evaporated milk than was present in the raw milk. Since certain studies in our laboratory differed from the results presented by Daniels, and because she had used repeated assays on the same animals but had not employed an improvement we had reported for our basic ration,[3] we have thought it advisable to investigate further the effect of the process of evaporation upon vitamin B_1.

Fresh raw milk was immediately iced and delivered by special messenger together with evaporated milk prepared from the same lot. By careful refrigeration the raw milk was kept sweet for the duration of the test. The evaporated milk was stored at room temperature.

Sixty-gram rats were fed the following ration: 15.0% vitamin-free casein, 15.0% dried autoclaved liver,* 45.5% sucrose, 17.0% Crisco, 3.0% cod liver oil and 4.5% modified Wesson's salts. After

[1] Daniels, A. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 212.

[2] Schlutz, F. W., and Knott, E. M., *J. Nutr.*, 1936, **12**, 583.

[3] Schlutz, F. W., and Knott, E. M., *J. Biol. Chem.*, 1937, **119**, lviii.

* Fresh hog liver was ground and autoclaved for 5 hrs at 120°.

22-25 days when a rat had been depleted of its vitamin B_1 reserve and was losing weight slowly but steadily, it was given a daily supplement of the material to be assayed. The dose was continued for 10 days, the amount being changed from day to day, if necessary, to maintain a gain of about 2 g per day. After administration of the dose was completed a rat was continued on the unsupplemented basic ration until it was again losing weight. The total dose which had been administered was then divided by the total grams of gain to obtain a growth unit for calculating the vitamin B_1 content. Crystalline thiamin was used as a standard control of the assay technic. For the sake of comparison with Daniels' results, certain groups of rats were given autoclaved whey in the basic ration instead of the autoclaved liver, and some rats were used for second and third assays when they had again become depleted after the first assay. As a further control, the curative technic as described by Supplee and Bender[4] was used to assay one lot of evaporated milk.

Results are reported in Table I. The raw milk was found to contain from 92 to 117 International Standard Units of vitamin B_1 per quart. The evaporated milks prepared from these raw milks contained 61 to 93 units per reconstituted quart, indicating losses of 34, 24, 21 and 20% respectively. There was no difference observed in the vitamin B_1 content of the irradiated and non-irradiated milks which had been prepared from the same lot of raw milk.

Remarkably comparable results were obtained when one lot of evaporated milk (M4591) was assayed by both the growth and curative technics. In the positive controls for these 2 technics, an average of 1.45 γ thiamin gave one gram of growth, while an average of 0.56 γ was needed to maintain a "cure" of one day. With the milk assays, averages of 3.83 and 1.50 cc were required respectively for the 2 technics, therefore 2.64 cc of milk were equivalent to one gamma of thiamin when growth was the criterion while 2.68 cc of milk were equivalent to one gamma of thiamin when the curative technic was employed.

When pure thiamin was administered as a supplement to the basic ration containing autoclaved whey, 2.38 γ were needed for a gram of growth in contrast to the 1.45 γ required with the liver ration. This poorer growth on whey was apparently due to a deficiency of the ration for some factor of the vitamin B-complex other than thiamin, since it was impossible to maintain growth with thiamin supplements to whey during second assay periods

[4] Supplee, G. C., and Bender, R. C., *Ind. and Eng. Chem.*, 1938, **10**, 636.

TABLE I.

Raw Milk				Evaporated Milk						
Date	No. of animals	cc milk per γ B_1	I.S.U. per qt	No.	Months since evaporation	No. of animals	cc milk per γ B_1	I.S.U.	% destroyed	Remarks
9-'38	9	3.01	105	M4591	0	8	1.99	80	24	Assayed with regular liver ration.
				M4591	2	6	2.50	63	40	Assayed with whey ration—first depletion.
				M4591	2	6	2.32	68	35	Assayed with whey ration—second depletion.
				M4591	2	6	2.29	69	34	Assayed with whey ration—third depletion.
				M4591	4	8	2.64	60	43	Assayed by regular growth technic.
				M4591	4	6	2.68	59	44	Assayed by curative technic.
6-'38	10	3.45	92	N6685	0	9	2.60	61	34	
10-'37	11	2.70	117	N4101	0	11	1.72	92	21	Irradiated
				N4101	8	7	3.24	49	58	"
10-'37	11	2.70	117	409N1	0	10	1.68	93	20	Non-irradiated
				409N1	8	6	3.62	44	62	"
				M6711	22	9	3.75	42		Assayed by regular technic.
				M6711	22	4	3.80	42		Assayed with a thiamin supplement added to the milk.

with the same animals. The whey ration therefore cannot be used indiscriminately for vitamin B_1 assays.

When milk M4591 was assayed by means of the whey ration, second and third 10-day growth assays showed 68 and 69 units of vitamin B_1 per quart as compared to only 63 units which were obtained with the first assay. Evidently evaporated milk contained the factor which the whey failed to supply, and thus made possible the uniformity in results for these repeated assays. Since the time interval between first and second assays was but a few days (as compared to the 3 weeks for initial depletion) the first assay was able to restore the rat's reserve of this factor sufficiently to make the second and third assays reliable.

Examination of the data for milk M4591 shows that changes had occurred in its vitamin B_1 content. When this milk was first brought to the laboratory and assayed it contained 80 International Standard Units of vitamin B_1. After 2 months the assay showed 68 units per quart, while still later assays gave only 59 units. The amount of vitamin B_1 destroyed had therefore increased from 24% for the original assay to 35% and 44% for the 2 later assays. This milk had been stored at room temperature. When milks N4101 and 409N1 were assayed after 8 months only 49 and 44 units were found, showing that decreases of 58% and 62% had occurred in the vitamin B_1 content of these 2 milks. A sample of milk (M6711) which had been stored in the laboratory for 22 months was assayed and found to contain 42 units of vitamin B_1.

The increased destruction of vitamin B_1 as evaporated milks are stored would appear to explain the differences in assay results which have been reported in the literature. Daniels who found 60% destruction studied milk purchased on the open market. Samuels and Koch[5] who found 16-20% destruction studied milk obtained directly after evaporation and stored at 5°C for several months. Dutcher, Francis and Combs[6] who reported only slight or negligible destruction prepared their own evaporated milk at intervals of only 3 weeks, and preserved it by refrigeration.

Further work is now in progress to determine just what change has taken place in the thiamin content of the milk, and to determine also to what extent pH, temperature, and time affect this change.

[5] Samuels, L. T., and Koch, F. C., *J. Nutr.*, 1932, **5**, 307.

[6] Dutcher, R. A., Francis, E., and Combs, W. B., *J. Dairy Sci.*, 1926, **9**, 379.

10483

Estimation of Erythrocytes from a Blood Smear by a "Dry Chamber" Method.

EMIL MARO SCHLEICHER. (Introduced by R. W. Keeton.)

From the Department of Medicine, University of Illinois, College of Medicine, Chicago, Ill.

If one could make an accurate red blood cell count from a smear, it would save time in manipulating the diluting pipette, hemacytometer, and avoid errors arising from sampling, diluting and chance distribution of the cells in the chamber.

The feasibility of estimating the erythrocytes from a blood smear was suggested by a former associate.* The "Dry Chamber" method reported briefly in this communication makes this possible. It has been in use for the past 2 years and is based on 2000 red blood cell counts, checked by the hemacytometer method, using standardized pipettes (Bureau of Standards). For the purpose of discussion the method may be presented under 4 headings.

I. *Making the Standard Smear.* For those not accustomed to making micro-slide preparations it is recommended that oxalated blood be drawn up to the 0.5 mark of a red blood cell dilution pipette, expelling the sample on a slide and then spreading the blood with a sharp cornered counting chamber cover glass, size 20x26,† held at an angle of 45°. The margin-free smear should be about 40 to 50 mm in length, and obviously 20 mm in width. When one has learned to judge the proper size of the drop, successive preparations will vary but little from each other. One may now use finger blood and proceed as is customary in making a red blood cell count. Wipe off the first drop. Permit the second drop to approximate the standard size. Pick up the blood sample with the 20 mm edge of the spreader. Apply to the slide and allow the blood to spread to a uniform layer along the edge of the cover glass. Push the spreader with an even, quick, sure movement towards the other end of the slide. The proper smear should be margin-free, devoid of coagulum, fibrin threads, waves or foamy appearance. This film approximates the volume of whole blood used in the red cell dilution pipette and if the second drop is collected the cellular quantities should be the same by either

* Suggested by Dr. E. A. Sharp, Detroit, Michigan.

† Made to specifications by Thomas and Company, Philadelphia.

method. The length and width of the blood smear assures an even distribution of the cellular elements. The air dried smear is stained, and air dried.

II. *Adjustment of Microscope.* The optical setting given below results in a constant which permits the estimation of the erythrocytes similar to that of the hemacytometer.

Optical Setting.‡

(a) Eye piece 10x.
(b) Tube length 160 mm.
(c) Oil objective 1/12.
(d) Whipple's ocular micrometer disk 7x7 mm square.§

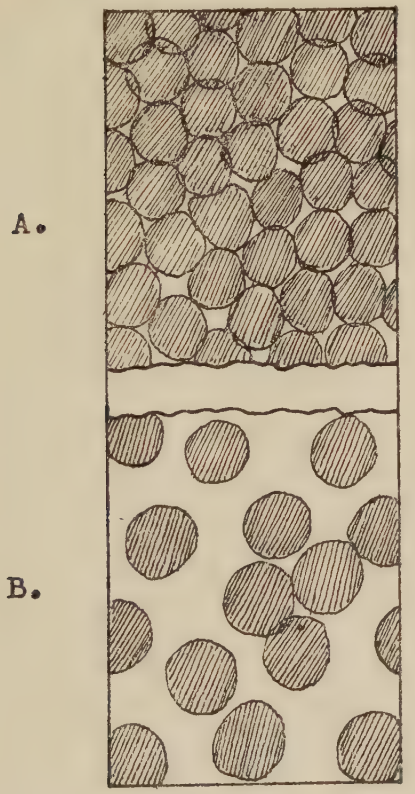


FIG. 1.
Normal Blood.
A. Minimum boundary pattern.
B. Maximum boundary pattern.

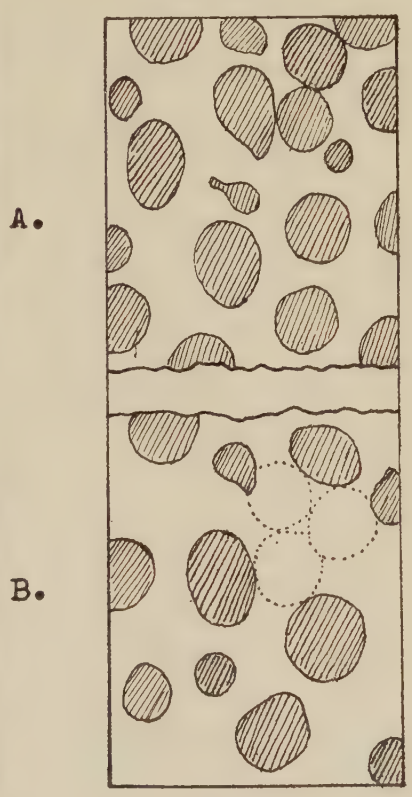


FIG. 2.
Pathologic Blood.
A. Minimum boundary pattern.
B. Maximum boundary pattern.
(The average cell is indicated by the interrupted lines)

‡ Zeiss, Standard Routine Microscope.

§ Standard Methods f. the Exam. of Water and Sewage, Am. Health Assn., 8th edition, pp. 178-79, 1936.

III. *Determination of the "Usable Area".* Cover the blood smear with a thin film of immersion oil and examine microscopically under low power for the determination of the usable area and its boundaries. A usable area is defined as that portion of a standard smear which shows the cellular elements evenly distributed according to the following specifications:

Minimum and Maximum Boundaries of the Usable Area for Normal and Pathological Blood.

A. *Normal Blood* (Fig. 1). (a) Minimum boundary: The erythrocytes should touch or slightly overlap each other..

(b) Maximum boundary: The erythrocytes should not be more than one average cell diameter apart.

B. *Pathologic Blood* (Fig. 2). (a) Minimum boundary: The erythrocytes should not be more than one average cell diameter apart.

(b) Maximum boundary: The erythrocytes should not be more than 3 average cell diameters apart.

The above specifications for the boundaries should be observed in at least 14 out of 15 low power micrometer squares. When low power is used with the micrometer square *in situ,* the square will resolve about 15 times into the 20 mm width of the smear. The boundary limits are read off the vernier scale of the mechanical stage and the midpoint determined by adding the boundary figures and averaging them. For example: Minimum boundary at 40 mm; maximum boundary at 60 mm; midpoint is at 50 mm.

Bring the 50 mm mark opposite the "0" mark on the fixed small vernier scale. Change to the oil immersion objective and move it directly vertical to the edge of the smear. The upper side of the ocular micrometer square should be parallel with the edge of the smear. Move the objective vertically a distance of 10 mm toward the center of the blood film; thus, the *"optimal micrometer square"* of the usable area is established (Fig. 3).

The proper fixing of these boundaries is essential since the

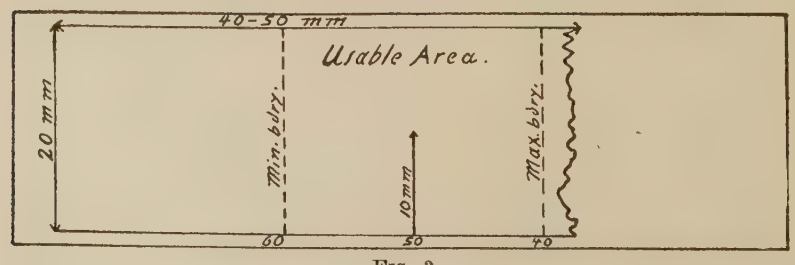


FIG. 3.

Margin free blood smear showing Usable Area with arrow point at optimal micrometer square.

magnitude of the numerical deviation from the counting chamber quantity depends on the proper placing of these limits.

Counting of the Erythrocytes. Count the erythrocytes within the boundary of the optimal micrometer square observing the same rule as used for the hemocytometer method. Mark the figure down. Count one large micrometer square adjacent to each side of the optimal micrometer square, thus counting 5 large squares consisting of 500 small squares in a cross-like fashion (Fig. 4-A). Average the total number of erythrocytes to obtain the number per ocular micrometer square (Fig. 4-B).

IV. *Comparison Between the Hemacytometer Unit and the Dry Chamber Unit.*

1. Hemacytometer Unit: In the counting chamber it should be recalled that the unit is 1 sq mm, subdivided in 1/25 sq mm. The later unit is made up of 16 small squares 1/20 mm each. Only the cells on 5/25 sq mm or 80 small squares are counted. The number of erythrocytes of undiluted blood is found by multiplying the RBC per 5/25 sq mm by 50x200, or 10,000, which is equivalent to adding 4 zeros.

2. Dry Chamber Unit: The ratio between the hemacytometer unit 1/25 sq mm and that of the ocular micrometer square is 1 : 0.72 and is referred to in this paper as "Dry Chamber Unit" or D.C.U. This unit has been found to be constant in a series of 2000 red blood cell counts done by both methods. In order to find the approximate equivalent erythrocyte number per 1/25 mm the number per micrometer square is multiplied by the D.C.U. 0.72, the resulting number is again multiplied by 5 to obtain the sum of

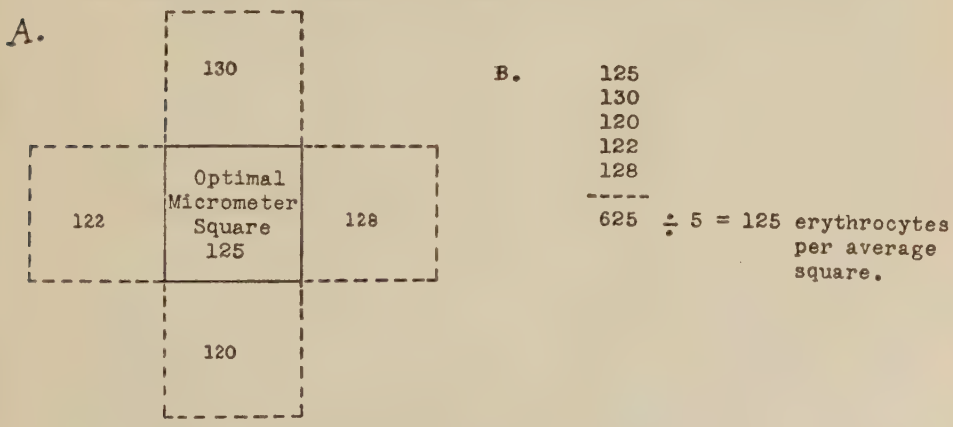


FIG. 4.

A. Arrangement of the additional fields to be counted. The number within the squares representing erythrocytes.

B. Method of arriving at the erythrocytes per average square.

TABLE I.
Comparison of Erythrocyte Counts Made by Hemacytometer and Dry Chamber Methods.

Diagnosis	Hemacytometer R.B.C. in mill.	Dry chamber R.B.C. in mill.	Experimental error of dry chamber
Pernicious anemia	1.360	1.368	+ 8
Sickle cell anemia	1.950	1.944	— 6
Acute hemorr. anemia	2.610	2.628	+18
Cooley's anemia	3.342	3.348	+ 6
Cong. elliptocytosis	3.730	3.708	—22
Cong. hemol. icterus	4.325	4.325	0
Chron. myel. leukemia	4.740	4.752	+12
Normal	5.040	5.040	0
Pern. anemia after liver therapy	6.130	6.105	—25
Polycythemia vera	8.750	8.712	—38

erythrocytes per 5/25 sq mm. To the product add 4 ciphers, as is done when the counting chamber sum of 5/25 sq mm has been determined. For example: RBC per average micrometer square is 125 erythrocytes; then $125 \times 0.72 \times 5 = 450$ cells, equivalent to 5/25 sq mm on the counting chamber. Add 4 ciphers to obtain the equivalent number of erythrocytes per cubic millimeter, or X number of erythrocytes $\times$ $0.72 \times 5 \times 10{,}000 =$ RBC equivalent to mm^3.

A total of 2000 comparative counts disclosed that the number of cellular elements found within the optimal square does not depend upon the size, shape, adhesiveness, nor specific gravity, but that their distribution in the standard smear is proportional to their mass.

The quantitative error of the Dry Chamber method is within $\pm 200{,}000$ permitted for the hemacytometer counts (Table I).

Summary. A method is described that permits estimation of erythrocytes from a blood smear. A specific optical setting is used yielding a ratio of the "Dry Chamber Unit" to the hemacytometer unit of 1: 0.72. On the blood smear a usable urea is determined. Its boundaries are described. The midpoint fixes the position of the Optimal Micrometer Square. Within this area the erythrocytes are counted in the same manner as done for the hemacytometer method. The average number of erythrocytes of 5 ocular micrometer squares is used for the estimation of the equivalent RBC number found with the hemacytometer method. A total of 2,000 comparative counts have been made on bloods ranging from 720,000 to 8,750,000 cells per mm^3. The Dry Chamber Method yields totals that are comparable with that of the hemacytometer. The quantitative error of the Dry Chamber count is within $\pm 200{,}000$, the standard error allowed for the counting chamber.

10484

Assay of Adrenal Cortical Extract.

R. J. Schachter and M. O. Bebee, Jr. (Introduced by A. J. Carlson.)

From the Department of Physiology, University of Chicago.

The effectiveness of adrenal cortical extracts can at present only be determined by their ability to maintain the lives of completely adrenalectomized animals. The animals which have been used in assaying adrenal cortical extracts are the cat, dog and rat. The guinea pig, which makes an excellent animal for bioassay of adrenal cortical extract, has been neglected because of the anatomical difficulty in extirpating the right adrenal gland.

This paper reports a simple technic for removing the adrenal glands in the guinea pig, and discusses the advantages which are gained by the use of the guinea pig instead of the cat, dog and rat.

To our knowledge, Simmons and Whitehead[1] are the only authors who describe a technic for the adrenalectomy in the guinea pig. By their method a great deal of shock is caused to the animal because they crush the penultimate rib and draw the kidney and the adrenal out through the wound. This shock is avoided by the following technic:*

1. Animals which have been fasted for one day are anesthetized with ether and turned on their left side. The right side is shaved, and the following description is for a right adrenalectomy.

2. A straight line incision approximately one inch long is made parallel to and immediately under the last rib. The incision extends about a half inch medial to the end of the last rib.

3. The liver is packed up, and the intestine medially with one pack each.

4. With the finger retracting the kidney caudalwards, the peritoneum, covering the ventral surface of the kidney and the adrenal gland, is torn with forceps to free the caudad margin of the adrenal.

5. The lateral and craniad margins of the adrenal gland are freed by cautiously tearing the ligaments joining the gland laterally to the body wall, and cranially to the diaphragm.

6. The posterior portion of the adrenal is cleared by turning over the freed craniad portion, and then removing the connective tissue from the underlying body wall. The gland is now only attached to the vena cava.

---

[1] Simmons, H. T., and Whitehead, R., *J. Physiol.*, 1936, **88**, 235.

* The surgery is cinema recorded.

7. The ligament, lying between the body wall, the vena cava, and the adrenal gland, is put under tension by downward traction on the kidney. The ligament is picked up by forceps in the right hand, then while traction on the kidney is released, the ligament is picked up with another pair of forceps.

8. Anchoring the lateral portion of the ligament with the forceps in the left hand, continued vertical traction by the forceps in the right hand is exerted until the ligament is torn. (This method of freeing the gland is the essential feature of the operation. The connective tissue that is being torn must be visible at all times, otherwise the vena cava is endangered.)

9. The caudad pole of the gland may now be picked up by the capsule, and rotated so that the underlying vena cava is visible. Upward tension stretches the fibers covering the gland and the vena cava, thus the gland may be removed from the vena cava, leaving the gland attached only to the adrenal vein.

10. The adrenal vein is clamped for about 30 seconds with one pair of forceps, with another pair of forceps placed between the clamping forceps and the gland enough traction is exerted to remove the gland from the body.

11. The body wall is closed by suture and the skin is closed by clips.

Since the adrenal gland of the guinea pig is very fragile, care is taken not to handle the gland with any instrument.

The left adrenal gland is removed in similar fashion, but less care is required, since the left adrenal gland does not lie on the vena cava.

In this work 50 guinea pigs were used; 20 were used for controls and 30 were treated with adrenal cortical extract. The control animals died of adrenal insufficiency at an average of 4.5 days (extremes 3 to 7 days).

The assay of the extract is as follows: The right adrenal of a 400-600 g guinea pig was removed, and 2 weeks later the left adrenal was removed. The day following the removal of the second adrenal, enough extract was injected for a period of 10 to 12 days, so that the pig gained weight and was in an apparent normal state. Following the withdrawal of the extract, the pig died of adrenal insufficiency within 3 to 4 days. Necropsy never revealed any accessory adrenal cortical tissue.

A unit is considered that amount of extract which, when 1 cc is injected daily, will maintain 1 kg of guinea pig in an apparently normal state for 10 days. Usually 1 cc equals 45 g of fresh whole adrenal tissue.

The advantage is that, once an extract is assayed for one guinea pig, it applies to most guinea pigs. This is not true of dogs.

Although cats and dogs have practically no accessory adrenal cortical tissue, they do not lend themselves for bioassay as well as does the guinea pig, because (1) a great many cats following surgery, even of only one adrenal, refuse to take their food, and die of starvation or have to be fed by stomach tube; (2) dogs, because of their size, require large quantities of extract.

The advisability of using the rat is this assay work is very questionable, because there is considerable controversy as to the survival period of the rat following adrenalectomy. One group of workers[2-7, 13] claim that the majority of rats indefinitely survive adrenalectomy, while another group[8, 9, 10] find that the majority of rats will survive adrenalectomy for only a few days. The most recent trend seems to be that the survival period of adrenalectomized rats depends on their diet[11-16] and environmental condition.[14, 17-20]

Another disadvantage of the rat is that it requires very large doses of extract to keep it alive. Thus, Cartland and Kuizenga[21] found that the rat requires 22 times as much extract per unit weight as does the dog, while D'Amour and Funk[22] found that a 50 g rat requires as much extract as does a 15 kg dog.

---

2 Scott, W. J., *J. Exp. Med.*, 1923, **38**, 543.

3 Jaffe, H. L., *J. Exp. Med.*, 1923, **38**, 107.

4 Lewis, J. T., *Am. J. Physiol.*, 1923, **64**, 503.

5 Rogoff, J. M., and DeNecker, J., *J. Pharm. Exp. Therap.*, 1925, **26**, 243.

6 Wyman, L. C., *Am. J. Physiol.*, 1928, **86**, 528.

7 Marmorston-Gottesman, J., and Perla, D., *J. Exp. Med.*, 1932, **55**, 109.

8 Pencharz, R. I., Olmstead, J. M. D., and Giragossintz, G., *Science*, 1931, **72**, 175.

9 Freed, S. C., Brownfield, B., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1931, **29**, 1.

10 Martin, S. J., *Am. J. Physiol.*, 1932, **100**, 180.

11 Rubin, M. I., and Krick, E., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 228.

12 Kutz, R. L., McKeown, T., and Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 331.

13 Gaunt, R., Tobin, C. E., and Gaunt, J. H., *Am. J. Physiol.*, 1935, **111**, 321.

14 Agate, F. J., and Zwemmer, R. L., *Am. J. Physiol.*, 1935, **111**, 1.

15 Cleghorn, R. A., Cleghorn, S. M. M., Forster, M. G., and McVicar, G. A., *J. Physiol.*, 1936, **86**, 229.

16 Swann, H. G., *Am. J. Physiol.*, 1937, **118**, 798.

17 Wyman, L. C., and TumSuden, C., *Am. J. Physiol.*, 1929, **89**, 362.

18 Hartman, F. A., Brownell, K. A., and Crosby, A. A., *Am. J. Physiol.*, 1931, **98**, 674.

19 Firor, W. M., and Grollman, A., *Am. J. Physiol.*, 1933, **103**, 686.

20 Weiser, R. S., and Morris, E. R., *Endocrinol.*, 1936, **20**, 556.

21 Cartland, G. F., and Kuizenga, M. H., *Am. J. Physiol.*, 1936, **117**, 678.

22 D'Amour, F. E., and Funk, D., *J. Pharm. and Exp. Therap.*, 1938, **62**, 307.

The disadvantages of the cat, dog and rat as assay animals for adrenal cortical extracts may be circumvented by the use of the guinea pig, because the guinea pig has no accessory adrenal cortical tissue, the surgery is comparatively simple, and the adrenalectomized guinea pig requires comparatively little extract to maintain it in an apparent normal state.

Summary. The merits of the cat, dog, rat and guinea pig as assay animals for adrenal cortical extracts is discussed. The guinea pig is found to be the most satisfactory animal because (1) it is easy to operate and handle; (2) it has no accessory adrenal cortical tissue, and (3) it requires small amounts of extract to maintain it in an apparently normal state.

We wish to thank Dr. Carlson for his advice during the course of this work.

10485 P

Modification of Sexual Development in the Opossum by Sex Hormones.*

CARL R. MOORE.

From Hull Zoological Laboratory, The University of Chicago.

Sex hormone application modifies to some extent the embryonic development of the reproductive system in reptiles, birds and mammals.[1-7] Immature pouch young of marsupials might be expected

* This investigation has been aided by grants to the University of Chicago from the Rockefeller Foundation, and from Armour & Co. Grateful acknowledgments are made to Mr. C. Blair Coursen, General Biological Supply Co., for obtaining for me 11 female opossums with pouch young; to Drs. Gregory Stragnell and Irwin Schwenk, Schering Corporation, for hormones; to Dr. Dorothy Price and Mrs. Sallie Johnson for most excellent assistance.

1 Kozelka, A. W., and Gallagher, T. F., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1143.

2 Willier, B. H., Gallagher, T. F., and Koch, F. C., *Proc. Nat. Acad. Sci.*, 1935, **21**, 625; *Physiol. Zool.*, 1937, **10**, 101.

3 Wolfe, E., and Ginglinger, A., *Arch. d'anat., d'Hist., et d'Emb.*, 1935, **20**, 219.

4 Dantchakoff, V., *Bull. Biol.*, 1936, **70**, 241; 1937, **71**, 269.

5 Greene, R. R., Burrill, M. W., and Ivy, A. C., *Science*, 1937, **86**, 200; 1938, **87**, 396; **88**, 130.

6 Hamilton, J. B., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 570.

7 Raynaud, A., *Bull. Biol.*, 1938, **72**, 297.

to provide the most favorable objects for such study on account of direct accessibility of young for treatment. In these mammals one may apply hormones directly to the young and thus avoid the usual interferences with placental function and lactation caused by injections of hormones into pregnant mothers.

Twenty-four litters of opossum pouch young have been available, 8 of known date of birth, which have provided a normal series of 47 males and females, in age 1 to 100 days, and an experimental series of 40 males and females subjected to hormone treatments beginning on days 3 to 32 and ending on days 16 to 62. Testosterone, t-propionate, and estradiol have been applied by cutaneous ointment (for effectiveness see [8]) and the latter 2 substances also by subcutaneous injection. The opossum at birth is "sexually indifferent" with Wolffian ducts, functional urinary ducts—Müllerian ducts appear on day 3 and gonads are definitely testes and ovaries on day 5.

The experimental series reveals that male and female hormones evoke responses of both Wolffian and Müllerian ducts in pouch young of either sex by day 22; inhibitory responses of ducts have not been observed. Duct stimulation was evident in all treated young sacrificed on day 16. The most striking and unexpected modification has been the tremendous development of the Müllerian system in females treated with testosterone or t-propionate, and the less marked responses of Wolffian ducts of both sexes treated with estradiol. Table I reveals duct modifications of 8 hormone-treated animals. In each the diameter of the duct lumen was obtained from stained serial sections and does not express the great increase in the walls of the structures. Lumen diameters are given for normal ducts from similar aged untreated embryos. Thus, in the first animal, treated with an estradiol ointment by daily brush application on the skin from day 5 to 22, the greatest Wolffian duct lumen is .52 mm in comparison with .03 mm in an untreated male (increase of 17 times); the Müllerian duct lumen is .18 mm in contrast to a non-lumenate solid cord of cells in untreated males of same age. A striking result is the pronounced androgenic stimulation of Müllerian ducts in females, in contrast to males, despite similar applications in both sexes from day 3.

Prostate gland anlage do not appear in normal female opossums (present in males on day 16) but a female treated with androgen readily develops a prostate greatly in excess of similar aged un-

[8] Moore, Carl R., Lamar, J. K., and Beck, Naomi, *J. Am. Med. Assn.*, 1938, **111**, 11.

TABLE I.
Diameter of Lumen of Wolffian and Müllerian Ducts (in mm) from Stained Serial Sections of Opossum Pouch Young.

Treatment	Days		Wolffian Duct		Müllerian Duct	
			Normal control	Treated	Normal control	Treated
Estradiol Ointment	5-22	male	.03	.52	cord	.18
		female	cord	.60	.03	.22
Testosterone Propionate	10-21	male	.03	.04	cord	.01
		female	cord	.04	.01	1.05
	16-31	male	.03	.11	cord	.04
		female	cord	.05	.07	1.40
Testosterone	3-24	male	.03	.03	cord	cord
		female	cord	.06	.01	.30

treated males. A female treated with androgen from day 10 to 30 and sacrificed without additional treatment on day 62 shows enormously hypertrophied Müllerian ducts (coiled oviducts, numerous uterine glands, constricted cervix, and dilated lateral vaginal canals) and a large quantity of prostate gland tissue.

Gonad development has suffered little if any histological modification from treatment during early development and only gross size modifications are apparent; ovarian activity has continued despite tremendous stimulations of other structures by testosterone.

Contrary to typical free-martin responses noted by Lillie[9] in cattle, chemical androgens (1) although stimulating some retention of Wolffian ducts do not cause retention of their entrance into the urinogenital sinus; (2) stimulate, instead of inhibit, Müllerian ducts in females; (3) do not suppress early ovarian activity and (4) stimulate both male and female external genitalia.

Conclusions. Opossum pouch young, indifferent as to sex at birth, show stimulated responses of both sex ducts from treatment with ointments or injections of androgens or estrogens; duct inhibitions are not evident. Responses are revealed by day 16. Androgenic stimulation of Müllerian, and estrogenic stimulation of Wolffian, ducts are unexpected; female prostate induction occurs with androgenic treatment and male prostate inhibition with estrogenic treatment. Responses differ from those revealed in the free-martin.

[9] Lillie, Frank R., *J. Exp. Zool.*, 1917, **23**, 371.

10486 P

Relation of Methemoglobin to the Cyánosis Observed After Sulfanilamide Administration.

C. J. Watson, Irwin Vigness and W. W. Spink.

From the Divisions of Internal Medicine and Biophysics, University of Minnesota Hospital, Minneapolis.

Two explanations have been offered for the cyanosis often noted after sulfanilamide administration. The first is that the drug causes formation of methemoglobin, or (secondarily, because of the presence of sulphur) sulfhemoglobin.[1, 2] The second is that a black[3] or blue pigment[4] derived from sulfanilamide, occurs in the blood and produces cyanosis. We have confirmed Ottenberg and Fox's observation[4] that colorless sulfanilamide solutions are changed to a bluish purple when exposed to ultraviolet light. The results of the present study reveal, however, that a pigment derived from sulfanilamide is not directly responsible for the cyanosis. We are now convinced that the cyanosis due to sulfanilamide is caused in all instances by methemoglobin (rarely sulfhemoglobin). In order to detect methemoglobin in every case it has been necessary to employ a sufficient concentration of laked blood (1:5, tube thickness 2 cm; Zeiss grating spectrometer). With more dilute solutions we have often failed to detect appreciable concentrations of methemoglobin.

The concentration of methemoglobin in the blood has been measured by one or both of two methods in a series of 26 determinations. The first method was a spectrocolorimetric procedure such as used previously for measuring the concentration of porphyrin.[5] Blood laked with distilled water in varying dilutions of from 1:10 to 1:30 (depending upon the relative concentration of methemoglobin), was compared with a 1:100 dilution of the same blood in distilled water, in which all of the hemoglobin was converted to methemoglobin by addition of $K_3Fe(CN)_6$. (2-3 mg to 100 cc). The second method was spectrophotometric, such as employed by Heilmeyer;[6] certain modifications will be described in

1 Paton, J. P. J., and Eaton, J. C., *Lancet*, 1937, **1**, 1159.

2 Hartmann, A. F., Perley, A. M., and Barnett, H. L., *J. Clin. Invest.*, 1938, **17**, 699.

3 Marshall, E. K., Jr., and Walzl, E. M., *Bull. Johns Hopkins* Hosp., 1937, **61**, 140.

4 Ottenberg, R., and Fox, C. L., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 479.

5 Watson, C. J., *J. Clin. Invest.*, 1937, **16**, 383.

6 Heilmeyer, L., *Medizinische Spektrophotometrie*, Jena, G. Fischer, 1933, p. 103.

TABLE I.

Case No.	Cyanosis	Spectroscopic detection of Methemoglobin band (1:5 dilution)	% of Methemoglobin with relation to total Hb.		Free blood sulfanilamide in mg %
			Spectrocolorimeter	Spectrophotometer	
1	+++	+			6.8
2	++++	+			7
3	+++	+	7.3		5
4	++	+			4.9
5	++	+			17.5
6 (12-17)	+++	+	2.5		
(12-18)	+++	+	8.0		20.8
(12-19)	+++	+	13.1		
(12-20)	+++	+	8.5	7.8	18.8
(12-21)	++	+	5.0		15.9
7	++	+	8.5	9.5	17.5
8 (12-21)	++++	+	30.9		9
(12-21)*	0*	0			
(12-30)†	++++†	+	30.3		
9	+++	+	14.2	13.3	7
10 (2-11)	++	+	10.0	12.3	9.6
(2-11)*	0	0		0.5	
11 (1-26)	+++	+	24.0	18.5	9.1
(1-26)*	0	0			
(1-27)†	++	+		13.4	6.1
12	+++	+	18.0	21.5	8.7
13	++	+	8.5	11.3	13
14 (3-3)	++++	+	38.4	40.0	6.4
(3-3)*	0	0			
(3-4)†	++++				
15	0	0		1.0	13

* 30 minutes after intravenous injection of 1% methylene blue solution (8-20 cc).

†After resumption or continuation of the drug.

detail elsewhere. The results of the study are shown in Table I.

These results confirm the conclusions of Hartman and his associates,[2] as well as the recent findings of Wendel[7] that methemoglobin (or sulfhemoglobin) is the cause of sulfanilamide-induced cyanosis, and that methylene blue, given intravenously, rapidly abolishes the methemoglobinemia. We have seen but one instance of sulfhemoglobinemia, and in this case, methylene blue had no effect. This patient had been given sulfanilamide because of peritonitis incident to carcinoma of the colon, bowel obstruction and perforation.

It should be emphasized that the spectral distribution curves of the bloods examined for methemoglobin with the spectrophotometer, failed to reveal the presence of any other pigment.

[7] Wendel, W. B., *J. Clin. Invest.*, 1939, **18**, 179.

10487

Attempts to Demonstrate a Surface Antigen of Staphylococci and Specific Phagocytosis.

Wesley W. Spink. (Introduced by C. J. Watson.)

From the Division of Internal Medicine, University of Minnesota Hospital, Minneapolis, Minn.

Lyons[1] has recently reported the results of his studies on antibacterial immunity to the staphylococcus. He states that young infusion-broth cultures of toxigenic and non-toxigenic strains of staphylococci are encapsulated; old infusion-cultures contain mostly non-encapsulated cocci; the capsule is not present in young serum-grown cultures; capsules are no longer demonstrable after agitation on a mechanical shaker, but they withstand heating to 100°C for 5 minutes; young (2-hour) broth cultures when exposed to defibrinated blood are only phagocyted in small numbers in 15 minutes, as compared to overnight cultures which are phagocyted in large numbers. In other words, the young encapsulated cocci are resistant to phagocytosis. Lyons indicates that a marked phagocytosis of a young culture is related to a specific factor present in serum. It should also be pointed out that he has reported type-specific agglutinins present in the serum of rabbits immunized with young cultures, whereas immunization with old cultures resulted in a preponderance of group-agglutinins. No swelling of the capsules *("Quellung")* could be demonstrated in young encapsulated organisms when added to this type-specific serum.

Because little is known concerning antibacterial immunity in patients with staphylococcal sepsis, it appeared highly important to extend the observations of Lyons as to whether young cultures of staphylococci isolated from patients possessed a capsule that could be demonstrated morphologically, and whether these young organisms resisted phagocytosis. In the present study, attempts were made to demonstrate an encapsulated phase of several strains of staphylococci. Quantitative phagocytic studies were also carried out with the use of human leukocytes and young and old cultures of staphylococci. Thirty strains of pathogenic staphylococci were used; 27 of which were isolated from patients, and 3 were supplied to us by Dr. A. T. Henrici of the Department of Bacteriology, University of Minnesota. Eleven non-pathogenic strains were also studied, 6 of which were sent to us by Dr. George H. Chapman

[1] Lyons, C. V., *Brit. J. Exp. Path.*, 1937, **18**, 411.

of New York, and 5 of which were grown from human urines.* Stock cultures of all the strains were maintained on veal-infusion agar-slants kept in a refrigerator. Transplants were made every 3 weeks without any demonstrable loss of, or change in, pathogenicity of the strains. Suspensions of the organisms were obtained by growth in veal-infusion broth containing 0.05% glucose, as recommended by Lyons. Capsule-stains were carried out as described below. Phagocytic tests were done with the bloods obtained from 8 patients with staphylococcal sepsis, 4 normal adults, and 3 infants.

We were unable to demonstrate encapsulated staphylococci in either young or old cultures with Hiss's capsule stain,[2] Muir's modified stain,[3] with the method of Lyons,[1] and with 15% colloidal silver solution. Lyons then recommended the following revised procedure.[4] Smears were prepared from young 2-hour broth cultures, and dried in the incubator, but never flamed. Carbol-fuchsin was prepared by dissolving 0.025 g of basic fuchsin† in 3 cc of dehydrated alcohol, and then adding 22 cc of 5% solution of phenol in distilled water. The solution, after shaking well, was filtered before using. The dried smear was covered with the carbol-fuchsin solution, which was allowed to remain for one to 3 minutes. The stain was decanted, and then covered for 10 seconds with an aqueous solution of 2% potassium hydroxide. This was decanted, the smear *blotted* dry, and then Lœffler's alkaline methylene blue was added for 10 seconds. *At no time was the smear rinsed in water.* With this technic, a deposition of the carbol-fuchsin around the cocci was observed. The usual appearance was a deep blue coccus surrounded by a deep pink material, which might be interpreted as an encapsulated coccus. However, this staining reaction was not found to be specific for young cultures alone. Broth cultures that had stood at room temperature for 72 hours showed a preponderance of "encapsulated" cocci. Young cultures subjected to mechanical agitation for 30 minutes likewise were "encapsulated." Young serum-grown cultures did not show a specific deposition of dye around the cocci because the whole

* The pathogenicity of all the strains used was determined by biological and serological methods to be described elsewhere. The non-pathogenic strains were killed in large numbers by normal human blood.

2 Zinsser, H., and Bayne-Jones, S., *Textbook of Bact.*, 7th ed. revised, 1937, 1026.

3 Muir, R., and Ritchie, J., *Manual of Bact.*, 6th edition, 1913, iii.

4 Lyons, C. V., personal communication.

† "Soloid" basic fuchsin obtained from Burroughs Wellcome and Co.

smear retained the stain, but when the same serum-grown organisms were separated from the serum by centrifuging, washed 3 times in sterile distilled water, and resuspended in sterile broth, a deposition of the carbol fuchsin around the coccus was readily demonstrated. Smears of animal charcoal suspended in infusion-broth revealed the same deposition of pink dye around the dark blue particles of charcoal. We may conclude from these observations that while the deposition of fuchsin in direct contact with the staphylococcus might possibly be interpreted as demonstrating capsular material, the stain is not specific for this material alone.

The next step was to determine whether there was any quantitative difference in the phagocytosis by human leukocytes of a young broth-culture of staphylococcus and an old culture of the same strain. If the young culture possessed an encapsulated phase, one would expect fewer cocci phagocyted when compared with an older culture that was non-encapsulated. It was believed *a priori* that regardless of whether or not the majority of cocci in young cultures were encapsulated, a larger number of organisms from old cultures would be phagocyted because the young culture would be in the "lag phase" of growth when metabolic activity was at its height, whereas the old culture would be in the "phase of decline" and would contain some dead or dying organisms.[5] In this study, an "old" culture was a 16- to 18-hour growth in infusion broth, while a "young" culture was prepared by seeding 0.2 cc of the "old" culture into 10 cc of infusion-broth, and incubating it at 37°C for 2 hours. Phagocytic tests were done with human defibrinated blood as follows: 0.25 cc of blood was added to each of 2 pyrex test tubes. To one tube 0.05 cc of the "young" culture was added and to the other 0.05 cc of the "old" culture. The tubes were sealed in a gas-oxygen flame and rotated in the incubator for 15 minutes. They were then opened, and smears were made and stained with Wright's stain. Fifty consecutive polymorphonuclear neutrophilic leukocytes were counted, and the total number of intracellular cocci in each leukocyte showing phagocytosis was noted.

The results from a large number of phagocytic experiments indicate that (1) the leukocytes of human defibrinated blood will phagocyte essentially the same number of cocci from "young" cultures as from "old" cultures. Occasionally, an isolated experiment showed fewer cocci phagocyted from the "young" culture,

[5] Topley, W. W. C., and Wilson, G. S., *The Principles of Bacteriology and Immunity*, 2nd edition, 1937, 67.

but such observations were inconstant. (2) While the total number of intracellular cocci is the same, it would appear that slightly fewer leukocytes entered into the phagocytosis of "young" cultures. (3) The bloods of patients with staphylococcal sepsis showed no greater degree of phagocytosis of "young" and "old" cultures of homologous strains than the bloods of normal adults and infants. (4) Pathogenic strains possessing potent hemolytic and lethal exotoxins were phagocyted as readily as pathogenic strains producing little or no exotoxins. (5) There was no appreciable difference in the phagocytosis of pathogenic strains as compared with non-pathogenic strains. In Table I are presented representative phagocytic studies of pathogenic staphylococci.

TABLE I.

Source of Blood	Strain‡	Old Culture†	Young Culture†
		%	%
1. Pt. "S" (osteomyelitis)*	"S"	479- 98	505- 92
2. Pt. "A" "	"A"	628-100	444- 98
3. Pt. "K" "	"K"	471-100	346- 85
4. Pt. "Sh" "	"Sh"	806-100	813- 88
5. Pt. "L" (bacterial endocarditis)	"Co"	385- 85	231- 80
6. Pt. "Fr" (carbuncle)	"Co"	322- 94	418- 96
7. Pt. "O" (bacteremia)	"Co"	699-100	757- 98
8. Normal adult*	"S"	522-100	618- 90
9. " "	"A"	634- 98	547- 90
10. " "	"K"	581- 98	604- 85
11. Normal infant (3 mo.)	J13	408- 86	463- 74
12. " " "	"S"	743- 96	576- 84
13. " " (6 mo.)	J13	562- 96	630- 80
14. " " "	"S"	710- 98	531- 80

* Tests were done with the bloods of 1, 2, 3, 4 at the same time as 8, 9, 10.

† Total number of intracellular cocci in 50 consecutive leukocytes with percentage of leukocytes showing phagocytosis.

‡ Source of Strains:

"S," "A," "K," "Sh" from patients with staphylococcal sepsis.

"Co"—Strain producing highly potent hemolytic and lethal exotoxin obtained from Dr. A. T. Henrici.

J13—Julianelle Type A organism obtained from Dr. A. T. Henrici.

10488 P

Hormone Iodine in Mother's and Umbilical Cord Blood.*

J. F. McClendon and C. E. McLennan. (With the technical assistance of Ralph White and William Foster.)

From the University of Minnesota.

The view has been expressed that the thyroid hormone passes through the placenta but since thyroglobulin has a molecular weight of 700,000 and thyroxine is practically insoluble in water, we thought more evidence on this question desirable. We have, therefore, determined thyroid hormone iodine in mother's blood at the time of delivery and in infant's blood taken from the umbilical cord.

TABLE I.
Hormone Iodine, Micrograms per 5 cc of Blood.

Case	Mother's blood	Umbilical cord blood
1	.25	.16
2	.11	.15
3	.26	.17
4	.30	.14
4	.34	.13
5	.26	.20
6	.10	.19
7	.10	.10
8	.24	.11
9*	.34	.13
9	—	.13
10	.20	.17
11	.15	.12
12	.12	.15

* Mother has been taking desiccated thyroid for 4 months.

Table I indicates that mother's blood may be more variable in thyroid hormone than is new-born infant's blood. We do not intend this table to show the limits of variation but we believe that these values cast doubt upon the assumption that thyroid hormone passes freely through the placenta. If it passes through the placenta we think it passes very slowly and that no equilibrium is established. Perhaps the fetus derives its thyroid hormone from its own thyroid.

* Assistance in the preparation of these materials was furnished by the personnel of Works Progress Administration Official Project No. 465-71-3-36.

10489 P

Measurement of the Stroke Output of the Human Heart by Roentgenkymography.

ANCEL KEYS AND H. L. FRIEDELL.

From the Laboratory of Physiological Hygiene and the Department of Radiology, University of Minnesota, Minneapolis, Minn.

Measurement of the area of the frontal silhouette of the human heart as registered on an X-ray plate permits calculation of the total heart volume with an average error of less than ±5% (studies on "fresh" cadavers, Keys and Friedell[1]). By means of the multiple slit roentgenkymograph (*cf.* Johnson,[2] Roesler[3]), it is possible to trace the outlines of the living human heart in both (ventricular) systole and diastole of a single cardiac cycle (Keys and Friedell[4]). From measurement of these areas the volume of the heart in systole and in diastole can be calculated. The error in volume estimation from the frontal area is mainly a reflection of abnormality in the relation between frontal and anterior-posterior shape of the heart. Since these abnormalities must be reflected both in systole and in diastole, the error in the estimation of the difference of volumes should be relatively small.

The difference between the volume of the heart in systole and in diastole should be directly related to the stroke output if the valves allow no back flow. The precise numerical relation, however, could not be predicted *a priori* because the auricular and ventricular cardiac cycles are not absolutely simultaneous in phase. In addition, the difference between the areas of the systolic and diastolic kymogram outlines must be underestimated because the kymogram only registers excursions in one of the 2 planes of the surface of projection.

We have developed a procedure in which roentgenkymograms are made immediately before and after acetylene rebreathing experiments. By this means we obtain estimates of diastolic and systolic volumes of the heart from the kymograms and of the cardiac stroke output by the method of Grollman.[5] Since the 2

1 Keys, A., and Friedell, H. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 267.

2 Johnson, S. E., *Am. J. Roentgenol. Rad. Ther.*, 1937, **37**, 167.

3 Roesler, H., *Clinical Roentgenology of the Cardiovascular System*, Charles C. Thomas, Baltimore, 1937.

4 Keys, A., and Friedell, H. L., *Science*, 1938, **88**, 456.

5 Grollman, A., *Cardiac Output in Man in Health and Disease*, Charles C. Thomas, Baltimore, 1932.

kymograms always check very closely, the 2 types of measurement can be considered to be physiologically simultaneous.

With this procedure we have found that:

$$\text{cardiac stroke} = 0.64\ (\text{Area}^{1.45}_{\text{diastole}} - \text{Area}^{1.45}_{\text{systole}}).$$

In 22 experiments the correlation between calculated and measured stroke was +0.984, the mean discrepancy was ±5% and the greatest differences were +10 and —11% referred to the acetylene method as standard. When duplicate determinations were made on the same subjects the averages agreed even more closely, the maximum error being only about ±7%. Typical results are shown in Table I.

These results were obtained on normal subjects; similar results were obtained on patients with normal heart valves but with myxedema, hypertension, glomerulonephritis, etc. The subjects studied had systolic heart volumes ranging from 450 to 750 cc—that is, approximately the entire normal range for adults. The stroke volumes ranged from 24 to 92 cc.

Our equation for the relation of frontal projection area to volume is: $V = 0.63\ (A)^{1.45}$ (Keys and Friedell[1]). The equation found here for stroke volume indicates that the net stroke output is almost exactly equal to the maximum volume difference between systole and diastole. The discrepancy is so slight that we can legitimately modify our area-volume coefficient from 0.63 to 0.64 to simplify the calculations. But the total stroke output (pulmonary plus systemic circulation) must be exactly twice this value. Something close to this relation would be predicted for the reasons given in the second paragraph of this paper.

Comparison with the results of other workers and trials by ourselves indicate that results with the acetylene method when re-

TABLE I.

Typical Results from Simultaneous Roentgenkymography of the Heart and Acetylene Rebreathing with 2 Normal Subjects in Rest (but Not Basal). Numbers in brackets refer to the order of the kymogram plates. Areas in cm², volumes in cc.

Subject	Corrected Frontal Area		Volume = $0.64(A)^{1.45}$		O_2 Uptake cc/min.	A.V. O_2 diff. cc/L.	Cardiac Index L/min./m.²	Stroke Volume	
	Diast.	Syst.	Diast.	Syst.				Kymo.	Acet.
W.O.									
(1)	136.3	125.9	797.0	709.7	264	44.2	3.20	87.3	85.3
(2)	136.2	126.0	796.0	710.7				85.3	
A.Li.									
(1)	106.3	97.9	556.1	492.8	252	51.0	2.65	63.3	63.6
(2)	106.2	98.1	555.4	494.5				60.9	

peated under constant conditions at the shortest possible intervals frequently vary by ±5% or more. The excellent agreement between the kymographic and the acetylene-rebreathing methods is remarkable in view of this. It would appear that the kymographic method provides an estimate of stroke output at least as accurate as the acetylene method within the limits of heart size and output studied by us.

It should be pointed out that the kymographic method requires practically no coöperation on the part of the subject; it is only necessary that the breath be held for about 3 seconds during the exposure. However, there is generally some apprehension or excitement involved in placing the subject in position and minimal cardiac output under basal conditions is not attained until several experiments have been made. This is similar to but less marked than in the acetylene rebreathing procedure.

10490 P

Quantitative Measurement of Valvular Efficiency of the Human Heart.

ANCEL KEYS AND H. L. FRIEDELL.

From the Laboratory of Physiological Hygiene and the Department of Radiology, University of Minnesota, Minneapolis, Minn.

In the preceding paper it was shown that the stroke output of the heart may be measured accurately by calculation from the diastolic and systolic areas traced on a roentgenkymogram. Actually, this is a measure of the amount of blood expelled from the heart; in the subject with normal valves it equals the net amount of blood *circulated* by the stroke. In the patient with aortic or mitral regurgitation, however, we should expect that such equality would not hold and the amount of blood circulated would be less than the amount expelled from the heart by the amount of regurgitation.

The net amount of blood circulated may be measured by the acetylene-rebreathing method. We have found it possible to make what are essentially simultaneous roentgenkymograms and acetylene cardiac output determinations. Comparison of the two results with this procedure should give an absolute quantitative measure of the extent of the back leakage (or the efficiency) of the mitral and aortic valves.

TABLE I.

Results from Simultaneous Roentgenkymography and Acetylene Rebreathing in a Case of Aortic Regurgitation (Moderate Leak) at Rest (but Not Basal). Areas in cm^2, volumes in cc.

		Corrected Frontal Area		Volume = $0.64(A)^{1.45}$		O_2 Uptake cc/min.	A.V. O_2 diff. cc/L	Cardiac Index L/min./m^2	Stroke Volume		Leak cc	Efficiency %
		Diast.	Syst.	Diast.	Syst.				Kymo.	Acct.		
2/8	I	148.5	136.5	902	798	250	39.9	3.4	104	75.4	28.1	72.5
	II	148.5	136.6	902	799				103			
3/1	I	146.7	138.0	885	811	232	50.2	2.1	74	50.2	23.8	67.8
	II	148.4	141.0	910	836				74			

The equation we have developed for the calculation of stroke volume gives, by definition, an average efficiency of the valves of 100% when subjects with normal valves are studied in this way with the two procedures used simultaneously. We have applied this procedure to a series of cases of aortic, mitral, and double aortic and mitral regurgitation and invariably have obtained results which differ from the normal subjects in the expected way.

The subjects were selected on the basis of a clearly defined clinical condition and ability to coöperate in the acetylene rebreathing. None of the subjects was decompensated, though several were on the verge of decompensation or had been decompensated at some time. The clinical evaluation of the lesions in the various patients differentiated 3 groups: (1) minimal leaks, (2) moderate leaks, (3) severe leaks. The leaks in the first group were such as might be missed in a hasty physical examination. Patients in the second group were able to carry on the ordinary routine of professional or clerical work but were restricted in their ability to indulge in exercise. The patients in the third group were marked examples of aortic or mitral regurgitation and were very much restricted in activity but not bedridden.

The summarized data on a patient with a "moderate leak" are given in Table I as an example of the results. This patient was apprehensive in the first experiment in spite of the fact a dummy trial had been made on the previous day. The results illustrate our constant finding that the cardiac output, measured by either method, is almost always considerably smaller in a second experiment.

Table II presents in condensed form the valvular efficiency found in 17 experiments with patients with aortic or mitral leaks. The absence of overlapping is probably fortuitous, but in any case the 3 groups are clearly differentiated from one another and from results in 22 experiments on subjects without valvular leaks.

It will be noted in Table I that, in this case, the efficiency of the

TABLE II.

Estimated Efficiency of Mitral and Aortic Valves in Subjects with Normal Valves and in Patients with Varying Degrees of Regurgitation. Constant resting condition in all cases.

No. of Experiments	Clinical Evaluation	% Efficiency	
		Mean	Range
18	Normal	99	*91-110
5	Minimal leak	87	82- 89
8	Moderate "	72	58- 78
5	Severe "	51	45- 54

* 16 of the 18 cases showed apparent efficiencies not less than 94%.

valves was somewhat greater at the smaller stroke volume. Similar results were obtained in second subject with a larger leak who was also studied on different occasions when the cardiac output differed markedly. This suggests that the percentage of leak is not independent of the total cardiac output but that the leak is relatively smaller at the higher level of output. Such a finding is in agreement with physiological expectations. The heart rate is generally faster when the stroke volume is increased and hence the duration of diastole, in which regurgitation occurs, is shorter. These studies are being continued on a larger scale, but it already appears that this method provides an acceptable quantitative measure of aortic or mitral regurgitation in man.

10491 P

Behavior of Dogs after Complete Temporary Arrest of the Cephalic Circulation.*

CLARENCE DENNIS AND HERMAN KABAT. (Introduced by M. B. Visscher.)

From the Department of Physiology, University of Minnesota, Minneapolis.

The authors have previously reported a method of decerebration of the dog by means of cephalic vascular stasis.[1] The emphasis at that time was upon the use of the procedure as a method for preparing decerebrate animals. Since that time our interest has shifted to the evidence that could be gained concerning the resistance of various cells in the brain to temporary cessation of blood flow and the correlation of these changes with changes in the behavior of the animal.

The technic has been modified to the following form: Two days after laminectomy at the second cervical level, the animal is atropinized, and a metal tracheal tube is inserted orally. A blood pressure cuff is wrapped about the neck, and the pressure is raised quickly to 700 mm Hg, at which level it is maintained as long as vascular arrest is desired, artificial respiration being administered through the tracheal tube. The completeness of circulatory

* Assistance in the preparation of these materials was furnished by the personnel of Works Progress Administration Official Project No. 665-71-3-69.

1 Kabat, Herman, and Dennis, Clarence, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 864.

arrest in the head is determined by ophthalmoscopic examination of the retinal vessels. With this technic, consciousness is lost in a very few seconds, the corneal reflex disappears in from 20 to 40 seconds, and spontaneous respiration ceases in from 40 to 90 seconds.

In 6 adult animals there was complete cessation of cephalic blood flow for periods of from 2 to 10 minutes. In the dogs subjected to 8 minutes or less of compression the lid wink returned in 5 to 8 minutes; in 2 animals treated for 10 minutes it did not return till later. The first gasp occurred in 1 to 5 minutes and satisfactory spontaneous respiration returned in 2 to 11 minutes, the slowest return being after the longest compression. Intermittent spontaneous, high-pitched vocalization, associated with vigorous running movements of all 4 limbs, with the dog lying on his side, often occurred during the first few hours. The 2-minute dog recovered consciousness within an hour, the 4- and 8-minute animals within 48 hours, and the 10-minute dogs failed to recover consciousness, even though surviving 4 and 6 days, respectively. Thus, as far as consciousness was concerned, the critical period of stoppage of cephalic blood flow in these dogs was between 8 and 10 minutes.

Among the 4 animals recovering consciousness, the dominant symptom was ataxia, the severity of which bore a fair relationship to the duration of compression. Early after compression these animals were comatose, later conscious but apathetic and dull. Either one or 2 days later, the first conscious movements were displayed in crawling in extremely ataxic fashion on the belly, the weight frequently being carried in the case of the fore paws on the dorsum instead of the pad of the foot. These animals showed a paucity of spontaneous movement for several days, but by the 10th day normal activity had returned, leaving only the ataxia as a residual symptom. Most of these dogs had been trained to sit up, shake hands, etc., prior to arrest of circulation; and all recovered these behavior patterns.

The dogs surviving 10 minutes of cerebral vascular stasis did not respond to visual, auditory, or olfactory stimuli. The animal would lie on its side, showing only slight extensor rigidity and occasional intermittent running movements of all 4 limbs often associated with vocalization. Postural and righting reflexes were absent. Simpler spinal and brain stem reflexes were normally responsive. If the dog's mouth were placed in milk, it would lap mechanically, slowing gradually and stopping after an ounce or two had been ingested.

In none of the animals here reported were convulsions observed. Consistent results with various fixed periods of cephalic vascular stasis have not been previously reported.[2,3] Our results are to a large extent predictable because we have accomplished complete stasis and eliminated anesthetic agents.

Histologic studies are in progress. These already indicate that the first cells to suffer from circulatory stasis are the Purkinje cells of the cerebellum, which correlates with the ataxic symptoms observed in our dogs.

10492 P

Influence of Asphyxia on Reciprocal Innervation.

A. VAN HARREVELD.

From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena.

In cats whose spinal cords had been asphyxiated for various periods of time, van Harreveld and Marmont[1] found that after recovery the hind legs could show an exaggerated extensor tone which usually stayed until death (in some experiments 3 weeks in total). Considering that after this time the acute effect of asphyxia will have disappeared, it was concluded that the high extensor tone is caused by a more or less selective damage of an inhibiting system present in the cord which normally keeps the tone in check.

A further study of these phenomena was made. A period of asphyxia for 30 minutes was usually followed within a few hours by the development of high extensor tone without abolishment of the flexor reflex. However, the effect of pinching the foot was not the same in all animals; sometimes it caused a regular flexor reflex with flexion in ankle and knee; in other experiments it caused, after the flexion, an extension of the leg, and in a few cases this stimulus caused instead of flexion an increase of the extensor tone.

When the contractions of the *M. tibialis anterior* and the triceps group were recorded simultaneously, it was often seen that stimulation of the *N. peroneus superficialis* caused a contraction in both of these antagonists. In other animals it was observed that the stimula-

2 Pike, F. H., Guthrie, C. C., and Stewart, G. N., *J. Exp. Med.*, 1908, **10**, 490.

3 Gildea, E. F., and Cobb, S., *Arch. Neurol. and Psychiat.*, 1930, **23**, 876.

1 Harreveld, A. van, and Marmont, G., *J. Neurophysiol.*, 1939, **2**, 101.

tion of the sciatic nerve caused a contraction in the quadriceps muscle of a Sherrington preparation instead of inhibiting the tone in this muscle.

By Sherrington's scheme for reciprocal innervation these observations can be best explained. Since, according to this scheme, the extensor motor neurones are inhibited during the flexor reflex, the simultaneous stimulation of afferent nerve fibers for extensor reflexes (*e.g.*, for the extensor thrust) running in the stimulated branch of the sciatic nerve, normally remains without effect. If the internuncial neurones transporting the impulses inhibiting the extensor motor neurones were damaged, it must be expected that the flexor and the extensor reflexes would occur at the same time. Thus the abolition of reciprocal innervation by asphyxia strongly supports the assumption that asphyxia damages the inhibitory neurones more severely than the excitatory ones.

10493

Effect of Heptyl Aldehyde on a Spindle-Cell Sarcoma.

WILLIAM G. CLARK. (Introduced by Eaton M. MacKay.)

From The Scripps Metabolic Clinic, La Jolla, California.

Strong[1] recently reported an inhibitory effect of heptyl aldehyde on spontaneous mammary tumors in mice and later[2] in dogs. He reported an inhibitory effect on the growth-rate of the mouse mammary tumors with complete regressions, gross and histological alterations, especially liquefaction of the tumors and high percentages of regressions in dog mammary tumors. In the mice he administered the heptyl aldehyde in the stock diet but directly injected small amounts of the undiluted aldehyde into the dog tumors. An attempt was made to extend Strong's results using a transplantable sarcoma in the rat.

The tumor used was a spontaneous sarcoma found in the liver of a female rat. It was highly malignant, of the spindle-cell type and retained its original vigor through the tenth passage in our particular strain of rats.[3] Tumor passage was afforded either by subcutaneous injection through a trochar of a single piece or a macerate or intraperitoneally by injection of a fine macerate in Tyrode's

[1] Strong, L. C., *Science*, 1938, **87**, 144; *Am. J. Cancer*, 1938, **32**, 227.

[2] Strong, L. C., and Whitney, L. F., *Science*, 1938, **88**, 111.

[3] MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1927, **83**, 179.

TABLE I.

Exp.	No. Rats	Sex	Treatment	Body wt in g Days after transplantation 0	3	9	18	Death	Tumor dimensions in mm on 9th day	Days survived	Tumor wt at death g	No. of tumors showing Necrosis	Liquefaction
1	3	♂	Controls	261						32			
	4	♂	Fed heptylaldehyde	265						27			
2	5	♂	Controls	72	95	128	147	150	33 x 14	25	30	3	1
	6	♂	Fed heptylaldehyde	83	108	136	142	140	29 x 16	25	18	5	3
3	6	♀	Controls	72	92	122	149	153	26 x 13	30	48	4	2
	6	♀	Fed heptylaldehyde	82	108	125	146	146	25 x 15	25	24	6	3

solution through a large needle. Tumors which developed at a subcutaneous site were localized large lumps, soft to palpation and at death frequently exhibited central necrosis and consequent liquefaction. The intraperitoneal growth was very diffuse showing multiple metastases over the entire peritoneum.

Heptyl aldehyde (Eastman) is a fairly toxic substance. One cc by stomach tube was fatal to rats of 200 g weight in 4 hours, prostration occurring within 15 minutes. There was acute hemorrhagic inflammation of the entire gastro-intestinal tract. The pungent odor of the aldehyde could be detected even in the muscles of the extremities. Strong did not indicate the doses which he fed, but the doses which were given here were as large as can be given without appreciably altering the food intake. Typical experiments are summarized in Table I. In Experiment 1 a saturated aqueous solution of heptyl aldehyde (about 0.1%) was administered by stomach tube in a dose of 1 cc per dcm^2 of body surface twice daily. In Experiments 2 and 3 the aldehyde was fed in the food in a concentration of 1%. The intake of the stock diet[3] was about 10 g per rat per day. These rats were 2 months old and all transplants were made subcutaneously between the shoulders.

It is obvious from the data presented that the heptyl-aldehyde-fed animals showed no greater tumor regression or liquefaction or survival time than the controls; although, the tumors of the treated animals weighed definitely less than those of the controls. Other experiments in which the aldehyde was administered in the drinking water in larger quantities confirmed this conclusion. The direct injection of small amounts up to toxic doses of the aldehyde directly into growing tumors was also without any influence.

Summary. Heptyl aldehyde was administered to albino rats by stomach tube, mixing with the diet, in the drinking water and injected directly into tumors. No effect was apparent on a malignant, transplantable spindle cell sarcoma with respect to resistance to growth of the tumor, survival time of the rat and regression or liquefaction of the tumor. The tumors of the rats receiving heptyl aldehyde weighed less at death in spite of the similar survival times.

10494

Evaluation of Wet and Dry Weight Bases for Expression of Respiratory Rate *in vitro*.*

JOHN FIELD, 2D, HARWOOD S. BELDING AND ARTHUR W. MARTIN.
From the Department of Physiology, Stanford University.

Much of the recorded data on tissue respiration *in vitro* have been calculated on a dry, rather than a wet, weight basis on the assumptions that dry weighings are more accurate and that dry weight, multiplied by 5, gives the wet weight with sufficient accuracy.[1] Because of the fundamental consequences of these assumptions, we have undertaken to determine their validity.

Thirty-three virgin, post-pubertal albino rats, 15 males and 18 females, belonging to 7 litters of Slonaker-Wistar stock, were used. Age range was 98 to 120 days, mean age 107.7 days, coefficient of variation of age, 6.09%. This limited range was chosen to minimize the decrease with age of oxygen consumption[2-5] and of tissue water content,[6] reported for this and other species. Tissue respiration was determined by the Warburg method,[1] with the several precautions suggested by Dixon,[7] in a water bath at 37.5° ±0.01°C. In every instance (317 cases) both wet and dry weight as well as oxygen consumption were ascertained.

Comparison of the variability of respiratory rate calculated on the wet and dry weight bases. The symbol Q_{O_2}, denoting oxygen consumption in ml, N.T.P., per g per hour, is commonly used to express respiratory rate *in vitro*. If the difference in accuracy of wet and dry weighings is critical, the variability of Q_{O_2} would be greater when computed on the basis of the less accurate weighing, other things being equal. To determine whether this were so, the coefficients of variation of Q_{O_2} were calculated for the organs and tissues which contribute most, in a quantitative sense, to the resting

* Supported in part by a grant from the Rockefeller Fluid Research Fund of the Stanford University School of Medicine and in part by aid furnished by the National Youth Administration.

1 Warburg, O., 1926, *Uber den Stoffwechsel der Tumoren*, Berlin.

2 Boothby, W. M., Berkson, J., and Dunn, H. L., *Am. J. Physiol.*, 1936, **116**, 468.

3 Davis, J. E., *Am. J. Physiol.*, 1937, **119**, 28.

4 Pearce, J. M., *Am. J. Physiol.*, 1936, **114**, 253.

5 Field, J., 2d, Tainter, E. G., Martin, A. W., and Belding, H. S., *Am. J. Ophthal.*, 1937, **20**, 779.

6 Adolph, E. F., *Physiol. Rev.*, 1933, **13**, 336.

7 Dixon, M., 1934, *Manometric Methods*, Cambridge.

TABLE I.

Group numbers denote order of descending value of mean wet/dry ratio.
Column 1. No. of cases.
Column 2. Mean value of wet/dry wt ratio.
Column 3. Standard error of mean wet/dry wt ratios.
Column 4. Coefficient of variation of mean wet/dry wt ratios.
Column 5. Coefficient of variation of Qo_2 on wet wt basis.
Column 6. Coefficient of variation of Qo_2 on dry wt basis.

Group No.	Organ Preparation	1	2	3	4	5	6
V.	Skeletal Muscle:				%	%	%
V.	Diaphragm	90	5.08	.085	15.9	18.2	22.2
V.	Abdom. obliques	26	5.26	.139	13.5	20.5	26.2
V.	Semimembranosus	20	5.39	.213	16.8	15.6	16.1
V.	Trapezius	17	4.90	.252	21.3	27.6	16.8
IV.	Intestinal Wall	21	5.60	.263	21.5	25.4	32.9
IV.	Heart Muscle	10	5.61	.262	14.8	23.0	13.7
III.	Kidney	26	6.03	.156	13.2	11.6	13.6
V.	Liver	24	5.07	.102	9.9	18.7	14.2
I.	Spleen	6	7.74	.550	17.4	11.0	10.5
II.	Lung	6	6.64	.264	9.7	7.3	9.0
	Cerebrum:						
II.	Cortex	12	6.53	.228	12.1	9.4	11.9
V.	Medulla	11	5.07	.204	13.4	17.4	22.3
VI.	Skin	15	2.54	.083	12.7	42.1	41.4
VII.	Bone:						
VII.	Femur	10	1.58	.048	9.6	22.6	27.5
	Rib	10	1.44	.047	10.1	48.2	45.7
	Cranium	6	1.39	.066	11.7	—	—
VI.	Cartilage	7	2.44	.285	30.9	34.2	29.1

metabolism. The several values so obtained are given in columns 5 and 6, Table I.

It was found by statistical analysis that the differences between the coefficients of variation of Qo_2 calculated on the wet weight basis (column 5) and the dry weight basis (column 6) would occur at least 9 times out of 10 in random sampling of a series in which no real difference existed.[8] Hence there is no significant difference in variability of Qo_2 on these 2 bases. An important theoretical consequence is that tissue water content is related as closely, in a statistical sense, to the factors determining Qo_2 as is the miscellaneous aggregate of substances making up the dry weight. Since comparison of respiratory rate *in vitro* is more readily made on the basis of wet weight, and since the variability of tissue Qo_2 is no greater on this basis, expression of Qo_2 as a function of wet weight will often be the procedure of choice. It seems probable that similar reasoning could be applied to other metabolic measurements *in vitro,* such as aerobic and anaerobic glycolysis.

[8] Fisher, R. A., 1934, *Statistical Methods for Research Workers,* London, pp. 117-120.

Wet-dry weight ratios (W/D ratios). The number of cases, mean value, standard error and coefficient of variation of the W/D ratio for the several organs and tissues are given in the first 4 columns of Table I. From inspection of these data it can be seen that the assumption that W/D = 5 holds quite well for skeletal muscle, cerebral medulla and liver, but not for the other organs and tissues. The significance of the differences in mean W/D ratios is shown in Table II. Here the several organs and tissues have been arranged in 7 groups in order of descending values of mean W/D ratio. An arbitrary scale of 0 to 4 (*c.f.*, legend, Table II) has been used to indicate the reliability of the differences observed.

The chance of random occurrence of the differences in mean W/D ratio between adjacent groups is always 1 or less in 10, while the differences between groups separated by an intervening group would occur by chance only 1 or less times in 100. It seems reasonable to regard these latter differences (denoted by 2 in Table II) and greater ones (denoted by 3 or 4) as due to other than chance variation. Therefore, under the conditions of our experiments, no single value can be assigned for the W/D ratio of the several organs and tissues of the post-pubertal albino rat.

TABLE II.

Evaluation of significance of differences in mean wet/dry wt ratio. The symbols 0 to 4 indicate, in increasing significance, the probability that the observed differences could not occur in random sampling.

Quantitatively these symbols have the following meaning:

0. Difference might occur by chance oftener than 1.5 times in 10
1. " " " " " 1 or less times in 10
2. " " " " " 1 " " " " 100
3. " " " " " 1 " " " " 1000
4. " " " " " 1 " " " " 10000

		Bone VII	Cartilage VI	Skin VI	Liver V	Muscle V	Cerebral Medulla V	Heart IV	Intestine IV	Kidney III	Cerebral Cortex II	Lung II
I	Spleen	4	4	4	4	4	4	3	3	2	1	1
II	Lung	4	4	4	4	4	4	2	2	1	0	
II	Cerebral cortex	4	4	4	4	4	3	2	2	1		
III	Kidney	4	4	4	4	4	3	1	1			
IV	Intestine	4	4	4	1	1	1	0				
IV	Heart	4	4	4	1	1	1					
V	Cerebral medulla	4	4	4	0	0						
V	Skeletal muscle	4	4	4	0							
V	Liver	4	4	4								
VI	Skin	4	0									
VI	Cartilage	3										

Summary. It has been shown in the case of the post-pubertal albino rat that: 1. The variability of Qo_2, based upon wet weight determinations, is no greater than when based upon determination of dry weight. 2. Organs and tissues differ significantly in respect to mean value of wet/dry weight ratio.

10495 P

Effect of Thyroxine on Eruption of Teeth in Newborn Rats.

D. KARNOFSKY AND E. P. CRONKITE. (Introduced by T. Addis.)

From the Department of Medicine, Stanford University, San Francisco.

The relation of the thyroid gland to the rate of differentiation of the organism is well known. An excess of the thyroid hormone causes the individual more rapidly to assume its adult structure, as, for instance, in the effect of thyroxine in the acceleration of metamorphosis in tadpoles, or in the more rapid appearance and uniting of ossification centers in the bones of various mammals. Conversely, a thyroid hormone deficiency decreases growth and differentiation and the organism tends to remain infantile. Although this tendency is well recognized and accepted, a more thorough study as to the effect of thyroxine on the rate of differentiation of the various organ systems of the mammal is indicated.

Hoskins[1] first began this type of study in young rats. They were injected starting at the third day after delivery with 0.1 mg of acetyl-thyroxine at 2- to 3-day intervals until the fifteenth day. She observed a more mature external appearance, the fur appeared more rapidly, the nails were longer, the shape of the skull narrower and epiphyseal activity was increased.

In the preliminary stages of a more detailed investigation of a similar problem we noted that the injection of thyroxine had a very marked and specific effect on the rate of eruption of teeth that was more noticeable than any other single change in the young rat. Since Hoskins began her injections on the third day and spaced them at 2- to 3-day intervals her quantitative observations were not as accurate as ours, but she states "Another difference to be seen at this period is the precocious eruption of the incisor teeth, which are visible at least 2 days earlier in the injected than in the control ani-

[1] Hoskins, M. M., *J. Exp. Zool.*, 1927, **48**, 373.

mals." We know of no further precise observations on the acceleration of the eruption of teeth in young rats by the use of thyroxine.

A litter of 5 newborn rats were injected with thyroxine-Roche, 0.05 cc (100 gamma of crystalline thyroxine) daily for 6 days. The injections were made subcutaneously in the dorsum of the rat. On the third day after birth, between 70 and 80 hours, it was noted that both the upper and lower incisor teeth had erupted. These rats lived to 6 days and then died of hyperthyroidism. Another group of 4 litters (20 rats) were subjected to the same dosage and it was consistently noted that the incisors appeared as small white spots just under the gingival mucosa at 50 to 60 hours after birth, and by 70 to 80 hours they were visibly erupted. In the controls (2 litters of 9 rats injected with 0.05 cc 10% alcohol) the teeth became visible to a similar degree between 120 and 130 hours (fifth day) and did not erupt until after 192 hours (eighth day). Donaldson[2] reports similar findings on the eruption of normal rat incisors. The acceleration of the rate of incisor teeth eruption from the eighth day to the third day is a distinct, easily observable and rapidly manifested action of thyroxine.

The dosage used in the above series was found to be extremely large. The injection of a single dose of 0.05 cc of thyroxine at birth in a litter of 5 rats caused the incisors to erupt between 80 and 90 hours; and 0.04 cc dose at birth in another litter of 4 rats caused the incisors to erupt at 90 to 96 hours.

We have not yet had an opportunity to standardize thyroxine dosage to time of teeth eruption except as indicated above, but the possibility of this observation lending itself to the standardization of the thyrotropic hormone suggested itself. Although the rat is known to be unusually refractory to the thyrotropic hormone the method was worth trying. A preparation of anterior pituitary of known thyrotropic potency* was used. 0.1 cc of the material was injected subcutaneously daily from birth until the eighth day in a litter of 5 rats. Doses of 0.2 cc were found to be fatal in newborn rats. No perceptible change in the rate of eruption of teeth was observed, and they appeared as in the controls between the eighth and ninth days. In this small series no response of the teeth to the thyrotropic hor-

2 Donaldson, H. H., *The Rat*, 1924, Wistar Inst. Anat. and Biol.

* The anterior pituitary used was a Squibb's preparation standardized at 10 Growth Units/cc. It was furnished by Dr. Evelyn Anderson of the University of California Medical School. She states its thyrotropic content is as follows: 0.1cc/day for 3 days to an immature guinea pig will cause a rise of 20% in the BMR at the end of that time.

mone was detected, and the use of the eruption of the rat tooth as a method of standardizing the thyrotropic hormone was shown to be of no value.

Summary. (1) The rate of the incisor teeth eruption in newborn rats is markedly accelerated by thyroxine; time of eruption varying with the dosage used. (2) The use of this observation as a method of standardizing the thyrotropic hormone was unsuccessful.

10496

Specificity of Graminae Pollens as Evidenced by Precipitin Reactions.*

ALBERT H. ROWE AND JACOB FONG. (Introduced by A. P. Krueger.)

From the Medical Department, Medical School, University of California, San Francisco.

Caulfeild[1] reported that guinea pigs can easily be sensitized with extracts of ragweed pollen precipitated with alum. During the last year we have produced hypersensitivity in guinea pigs with extracts of graminae pollens precipitated with alum and have demonstrated this state of hypersensitivity by the production of varying degrees of typical anaphylactic shock 3 to 5 weeks after sensitization of such animals.

In this paper we wish to report the results of specific precipitin tests of sera of such sensitized guinea pigs to extracts of their specific antigen pollens as well as to extracts of various other graminae pollens.

Pollen extracts were made according to Caulfeild's methods.[1] Groups of 5 guinea pigs each were sensitized with freshly prepared extracts of each of the following pollens: *Lolium perenne* (English rye grass), *Avena fatua* (wild oats), and *Bromus carinatus* (brome grass). In addition 3 animals were sensitized to *Cynodon dactylon* (Bermuda grass). One cc of extract was injected subcutaneously on 2 successive days to produce sensitization.† Anti-sera were ob-

* The authors thank Dr. Leonard Ginzton for suggestions given concerning experimental technic.

1 Caulfeild, A. H. W., *J. Allergy*, 1936, **7**, 451.

† The use of 2.5 cc or 5.0 cc of the same extract in one injection sensitized guinea pigs as well or possibly better in Caulfeild's experiments.

TABLE I.

Pollen Extracts Used as Antigens for Precipitin Reaction.

Serum from Animals Sensitized to	*Bromus carinatus* (brome grass)	*Avena fatua* (wild oats)	*Lolium perenne* (English rye grass)	*Cynodon dactylon* (Bermuda grass)	*Andropogon halepensis* (Johnson grass)	*Poa pratensis* (Kentucky blue grass)	*Poa annua* (walk grass)	*Dactylis glomerata* (orchard grass)	*Phleum pratense* (timothy)	*Elymus condensatus* (giant rye)	*Elymus triticoides* (slender wild rye)	*Festuca rubra* (red fescue)	*Anthoxanthum odoratum* (sweet vernal)	*Phalaris canariensis* (canary grass)	*Plantago lanceolata* (English plantain)	*Atriplex patula* (spearscale)	*Ambrosia psilostachya* (western ragweed)	*Artemesia californica* (coastal sagebrush)	*Pinus muricata* (tamarack pine)	*Pinus ponderosa* (yellow pine)	Saline
Bromus carinatus (brome grass)	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Avena fatua (wild oats)	—	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lolium perenne (Eng. rye grass)	—	—	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cynodon dactylon (Bermuda grass)	—	—	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Sera of each 5 animals sensitized to the first 3 pollens were tested separately with each of the 20 pollen extracts and similar results were obtained with all sera. The sera of 3 guinea pigs sensitized to *Cynodon dactylon* (Bermuda grass) gave similar results.

tained from the sensitized guinea pigs 4-5 weeks after the last preparatory injection of antigen.

Precipitin tests were executed with 5% freshly prepared alum-free pollen extract; the sera obtained from the sensitized guinea pigs were overlaid with the antigen, incubated and observed during a one-hour period.

The results as recorded in Table I show that the sera contained precipitins only for the specific grass pollen antigens with which the guinea pigs were sensitized and not for other graminae pollen extracts tested. The findings concerning the specificity of the precipitin reactions of graminae pollens agree with those of Parker[2] and Cromwell and Moore;[3] they also lend support to the clinical observations reported by Watson and Kibler,[4] Bernton,[5] Loeb and Petow,[6] Piness and Miller,[7] Rackemann and Smith,[8] Benjamins,[9] and Grubb and Vaughan[10] that desensitization with specific pollens is necessary to produce the best results.

Conclusions. 1. Sera of guinea pigs sensitized with extracts of certain graminae pollens contained specific precipitins only for the antigen used. 2. The antigen structure of graminae pollens appears to be specific. 3. These experiments justify the use of a specific pollen therapy.

---

2 Parker, J. T., *J. Immunology*, 1924, **9**, 515.

3 Cromwell, H. W., and Moore, M. B., *J. Allergy*, 1933, **4**, 347.

4 Watson, S. H., and Kibler, C. S., *J. A. M. A.*, 1922, **78**, 719.

5 Bernton, H. S., *J. A. M. A.*, 1924, **82**, 1434.

6 Loeb, L. F., and Petow, H., *Klin. Wchnschr.*, 1937, **6**, 57.

7 Piness, G., and Miller, H., *J. Allergy*, 1930, **1**, 483.

8 Rackemann, F. H., and Smith, L. B., *J. Allergy*, 1931, **2**, 364.

9 Benjamins, C. E., *Z. f. Hals-, Nasen-, u. Ohrenh.*, 1933, **33**, 153.

10 Grubb, G. D., and Vaughan, W. T., *J. Allergy*, 1938, **9**, 211.

10497

Heat Inactivation of Intracellular Phage Precursor.*

A. P. Krueger, T. Mecraken and E. J. Scribner.
From the Department of Bacteriology, University of California.

Krueger and Mundell[1] recently reported a method for demonstrating intracellular phage precursor. The essential step in the method is the preparation of "activated" suspensions of staphylococci; this is accomplished by growing the organisms in a heavily oxygenated medium. The activated cells are separated from the broth, resuspended in Locke's solution and are then maintained at 5°C for 2 hours before they are used. To demonstrate intracellular precursor 4 ml of activated cell suspension containing 5×10^8 bacteria/ml is added to 1 ml of phage diluted with Locke's solution to contain 1×10^9 activity units/ml. The mixture is kept for 5 minutes at 5°C and is promptly titrated for total phage content. The end titer is 2×10^9 activity units/ml, an increase of 500% in phage concentration.

Krueger and Scribner[2] supplied additional evidence for the existence of the intracellular precursor. They made use of the fact that staphylococci activated in the presence of Mn^{++} have a relatively low lytic threshold and require only small amounts of phage to induce lysis. Starting with a small amount of phage it was possible to transform the phage precursor in successive lots of staphylococci into phage and to obtain the newly formed phage free in solution by lysing the precursor-containing cells. The original phage added at the start of the experiment has been diluted at least 1 to one million without any reduction in plaque count or activity titer.

It seemed likely from data already available that the phage precursor is more thermolabile than the bacterial cell which produces it. In the experiments of Krueger and Fong[3] the relationships shown in Table I between bacterial reproduction and phage formation at various temperatures were observed.

It is evident that phage production has a temperature optimum of approximately 35°C. At 40°C the rate of phage production is only

* This work was supported by grants-in-aid from the Research Corporation and several interested friends.

1 Krueger, A. P., and Mundell, J., *Science*, 1938, **88**, 550.

2 Krueger, A. P., and Scribner, E. J., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 51.

3 Krueger, A. P., and Fong, J., *J. Gen. Physiol.*, 1937, **21**, 137.

TABLE I.
Rates of Bacterial Growth and Phage Production at Various Temperatures. From Krueger and Fong.[3]

Temperature	% increase in [Bacteria]/hr.	% increase in [Phage]/hr.
30°C	50	2000
35°C	125	8000
40°C	175	1250
45°C	150	Drops

one-sixth of the 35°C rate. At 45°C there is no longer an increase in [phage] but rather a definite drop. This last mentioned result conceivably could be due to inhibition of the intracellular precursor-producing mechanism at the higher temperature or to heat inactivation of the precursor after it is produced.

We wish to report here experiments performed to determine whether the precursor content of staphylococci could be inactivated at temperatures that would not kill the organisms. Suspensions of activated staphylococci were prepared as described elsewhere. For each experiment 40 ml of a Locke's solution suspension of activated organisms containing 5 x 10^8 cells per ml was placed in a thin-walled glass container. A thermometer was immersed in the suspension and

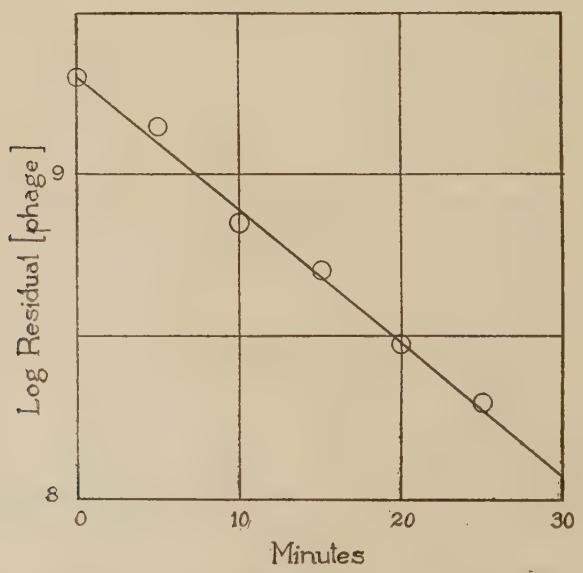


FIG. 1.

The rate of inactivation of intracellular phage precursor at 45°C. The ordinate represents the total phage formed when 4 ml of the heated sample was mixed with 1 ml of phage containing 1 x 10^9 activity units/ml. Each mixture was kept at 5°C for five minutes before titration. The points represent the average of six separate experiments.

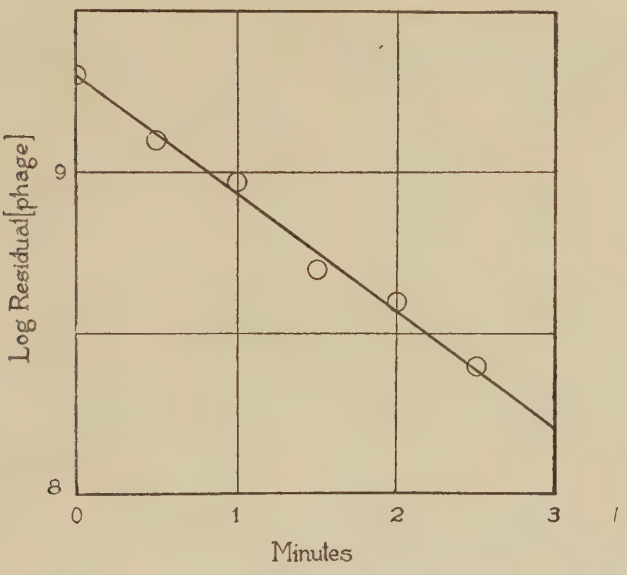


FIG. 2.
The rate of inactivation of intracellular phage precursor at 50°C. The points represent the average of five separate experiments.

the container was then placed in water at 70°C in order to attain the temperature of the experiment rapidly. As soon as the proper temperature was reached the container was transferred to a waterbath held at the desired temperature and samples were removed at short intervals to determine the number of viable organisms and the presence or absence of intracellular precursor in the bacteria. For the former purpose multiple samples were counted on agar pour plates after 36 hours of incubation at 37°C. For detection of precursor in the organisms each 4 ml sample was mixed with 1 ml of phage diluted in Locke's solution to contain 1 x 10^9 activity units per ml. The mixture was kept at 5°C for 5 minutes and was then titrated for total phage content by the activity method.[4]

Figures 1 and 2 summarize graphically the results of 6 experiments performed at 45°C and 5 performed at 50°C. After 25 minutes' exposure to 45°C the cells ceased to give a positive test for phage precursor. To obtain the same result at 50°C requires about 2½ minutes. During these periods of exposure the total viable cell count showed no significant reduction in any of the experiments; the essential data of the bacterial counts are summarized in Table II.

[4] Krueger, A. P., *J. Gen. Physiol.*, 1929-30, **13**, 557.

TABLE II.

Bacterial Counts During Heating of Activated Cell Suspensions at 45°C and 50°C. The values are all x 10^8 and represent averages of 3 dilutions prepared as agar pour plates and counted after 36 hours at 37°C.

45°C	Time of Sampling (Minutes)				
Exp. No.	0	15	30	45	60
1	4.75	4.85	4.47	4.19	3.19
2	5.28	6.25	6.40	4.47	2.54
3	6.05	6.46	5.43	2.30	1.53
4	7.15	6.1	5.81	2.42	
5	5.5	5.13	5.6	5.34	4.55
6	5.18	5.39	4.8	4.25	2.95

50°C	Time of Sampling (Minutes)					
Exp. No.	0	0.5	1.0	1.5	2.0	2.5
1	6.9	7.5	6.6	6.88	6.9	6.75
2	5.25	5.1	5.4	4.9	5.3	5.15
3	4.8	4.9	5.15	4.7	4.78	4.5
4	5.05	6.0	4.9	5.16	5.24	4.96
5	5.12	5.01	5.24	6.0	5.12	5.02

The velocity constants for the inactivation reaction were calculated from the equation:

$$d\ P/dt = k(P_o - P_i)$$

where P = [Precursor] at any time t, expressed as the total [Phage] formed upon the addition of 4 ml of activated cell suspension to 1 ml of Phage solution containing 1 x 10^9 activity units/ml.

P_o = Initial total Precursor/ml expressed in the same terms and P_i = Precursor/ml inactivated in time t.

For heat inactivation of precursor at 45°C k = 0.094; at 50°C k = 0.844. These values were substituted in the v'ant Hoff Arrhenius equation and μ, the critical thermal increment, was calculated to be 90,000.

μ for the destruction of phage precursor is of the order of magnitude characteristic of protein denaturation reactions in general and closely approximates the value reported for the heat inactivation of the homologous phage (101,000). The simplest assumption then would be that phage precursor is an intracellular protein similar to phage itself. However, it is possible that our experiments have not measured the actual rate of precursor inactivation but have followed the rate of denaturation of some entirely separate cell protein which when denatured alters permeability relationships and prevents access of phage to the cell's store of precursor. Whichever of these explanations proves to be correct it seems clear that the heat treatment of activated cells under controlled conditions results in loss of the phage-augmenting capacity characteristic of activated cells without entailing any significant number of cell deaths.

10498

Relation of Concentration of Virus to the Pathogenesis of Poliomyelitis.*

E. W. Schultz and L. P. Gebhardt.

From the Department of Bacteriology and Experimental Pathology, Stanford University, California.

To determine the route by which the virus spreads from the portal of entry to the medulla and cord, previous investigators have examined different regions of the central nervous system at varying intervals of time during the preparalytic period for the presence of virus. The results of these and related studies have established that poliomyelitis virus spreads axonally,[1] probably as an intraneural infection.

The work we wish to report represents an extension of these earlier studies in which the presence or absence of virus was merely determined and the amount present was not actually measured. Our investigations deal with the concentrations which are reached by the virus during the preparalytic period. They were prompted in part by certain observations on the kinetics of bacteriophagy which indicate that the lytic agent is at first produced in the absence of recognizable bacterial lysis and that actual lysis is not initiated until a certain critical concentration of the agent has been reached.[2] The mechanism of this prelytic formation of bacteriophage is not yet known and need not concern us here. In the studies here reported it was our purpose merely to determine the concentration of poliomyelitis virus which is reached in different regions of the central nervous system and to relate this, if possible, to the extent of neuronal damage sustained in the particular regions, the guiding hypothesis being that the production of virus and of nerve cell damage do not necessarily parallel each other and that certain neurons, although supplying a pabulum for the production of virus may, nevertheless, be relatively resistant to its action.

Part of our observations was made on intracranially inoculated animals; part on intranasally inoculated animals. All were sacri-

* Studies supported by Mary Hooper Somers Fund for Filtrable Virus Research.

1 Hurst, E. W., *J. Path. and Bact.*, 1929, **32**, 457; Fairbrother, R. W., and Hurst, E. W., *Ibid.*, 1930, **33**, 17; Hurst, *Ibid.*, 1133; Faber, H. K., and Gebhardt, L. P., *J. Exp. Med.*, 1933, **12**, 83; *J. Pediat.*, 1938, **13**, 1938; Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1938, **68**, 39.

2 Krueger, A. P., and Northrop, J. H., *J. Gen. Physiol.*, 1930, **14**, 223; Clifton, C. E., and Morrow, G., *J. Bact.*, 1936, **31**, 441.

ficed at varying intervals of time after the onset of the initial febrile temperature rise[3] and before the appearance of the usual recognizable symptoms of the disease. After exsanguinating the animal, individual specimens were removed with fresh sets of sterile instruments. Immediately after removal these were weighed and stored in 50% glycerine until the time of titration. In carrying out the titrations, the previously weighed specimens were ground thoroughly in a motorized grinder for one hour and made up to a 10% suspension in physiological saline. From this stock suspension further dilutions were made. The results presented below are expressed in terms of the *final dilution* of nervous material which proved infectious when injected intracerebrally into rhesus monkeys in 1 cc amounts. For the sake of orientation, we may say that in routine work pools of 5 or more glycerinated entire cords and medullæ from recently paralyzed monkeys and weighed after glycerination (glycerination for one month adds about 25% to the weight), generally prove infectious in dilutions as high as 1:4,000, less often in dilutions above 1:5,000 when injected intracerebrally in 1 cc amounts.

Our first observations were made on 2 intracerebrally inoculated monkeys. Each was inoculated with about 20 MID of virus. The first animal (C184) was sacrificed immediately after the onset of the febrile temperature rise, which was 4 days following inoculation. In this animal the thalamus proved infectious in a final dilution of 1:100, while dilutions of 1:10 of the olfactory mucosa, olfactory bulbs, medulla, cervical cord and the thoracic sympathetic ganglia failed to infect. Histological sections from these regions showed no deviation from the normal (olfactory bulbs were not examined). The second monkey (C183) was sacrificed 1 day following the onset of the febrile temperature rise, or 6 days after inoculation. In this animal the thalamus proved infectious in a dilution of 1:4,000; the medulla in a dilution of 1:3,000; the cervical enlargement of the cord in a dilution of 1:10, while 1:10 dilutions of the hippocampus, the lumbar enlargement and the abdominal and thoracic sympathetic ganglia failed to infect. Although the animal was inoculated intracranially, the bulbs proved infectious in a dilution of 1:10 (higher dilutions were not tested). Histological sections showed early perivascular round cell infiltration in the hippocampus, thalamus and medulla, with slight acidophilia of the nucleoplasm of some nerve cells in the medulla and cervical cord, but other than this there were no apparent changes.

[3] Kramer, S. D., Hendrie, K. H., and Aycock, W. L., *J. Exp. Med.*, 1930, **51**, 933.

Two of the animals on which our observations were made were inoculated intranasally. Both received 3 instillations of 10% pooled virus, all on the same day, each instillation being preceded by an acid phosphate lavage (pH 5). One of these animals (D457) was sacrificed immediately after the onset of the febrile temperature rise or on the third day following inoculation. In this animal the olfactory bulbs proved infectious in a dilution of 1:2,000 (the end point was not determined), the thalamus and uncinate gyrus in final dilutions of 1:100, the anterior perforated space and medulla in dilutions of 1:10, while 1:10 dilutions of the motor cortex and of the cervical enlargement of the cord failed to infect. Nasal washings, a suspension of finely ground olfactory mucosa and a suspension of the tonsils also proved non-infectious.† The tissue showed a beginning perivascular infiltration in the region of the anterior perforated space, hypothalamus, pons, medulla and cervical cord. Scattered nerve cells in these regions showed slight acidophilia of the nucleoplasm, but no retrograde changes associated with satellitosis or neuronophagia were observed. In the hypothalamus the cytoplasm as well as the nucleoplasm of a few nerve cells showed one or more small strongly eosinophilic bodies, resembling type B inclusions (Lentz A stain). Other nerve changes were not evident.

The second intranasally inoculated animal (D586) was sacrificed one day after the onset of the febrile temperature rise, or on the 6th day following inoculation. In this animal the olfactory bulbs were infectious in 9 different dilutions ranging from 1:25 to and including 1:10,000 (the endpoint was not determined); the hypothalamus in a dilution of 1:3,000; the thalamus, medulla and cervical enlargement of the cord in dilutions of 1:1,000; the lumbar enlargement of the cord in a dilution of 1:100, and the uncinate gyrus in a dilution of 1:10. Microscopically there was early perivascular round cell infiltration in the thalamus and this was more pronounced in the hypothalamus and medulla. No definite nerve cell changes were observed in the thalamus and hypothalamus. In the medulla a few nerve cells showed acidophilia of the nucleoplasm, but no definite retrograde changes nor evidence of satellitosis or of neuronophagia were observed. In both the cervical and lumbar enlargements of the cord only beginning perivascular round cell infiltration was observed.

We are unable to say anything about the histology of the olfactory bulbs in these animals since the entire bulbs were ground and used in

† Sabin and Olitsky[4] have recently also reported failure to demonstrate virus in the nasal mucosa of intranasally inoculated monkeys.

[4] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1938, **68**, 39.

the titration. Histological observations have, however, been made on the bulbs of a number of other intranasally infected and extensively paralyzed animals. These agree in the main with those recently reported by Sabin and Olitsky,[5] except that we have not observed extensive mitral cell involvement. At any rate neuronal damage here is less evident than in the anterior horns of the cord despite the high concentration of virus reached in the bulbs.

We have also titrated the cervical and lumbar enlargements of the cord of 2 intranasally infected monkeys (PMD623 and PMD 642A), sacrificed several hours after the onset of paralysis. In one of these (PMD623), which showed paralysis of all 4 limbs, the cervical enlargement infected in a dilution of 1:20,000, while the lumbar cord did so in a top dilution of 1:30,000 (however, 2 monkeys inoculated with the 1:10,000 dilution failed to develop the disease). Microscopic sections from the cervical and lumbar enlargements of the cords showed the usual extensive nerve cell changes and tissue reactions seen in early acute experimental poliomyelitis. In the other animal (PMD642A), which had developed paralysis of the arms, but not yet of the legs, the cervical enlargement of the cord infected in a dilution of 1:15,000, the lumbar in final dilution of 1:30,000. Microscopically, nerve cell changes were widespread in both the lumbar and cervical enlargements of the cord, but these seemed more advanced in the cervical enlargement where satellitosis and neuronophagia though early also seemed more evident.

In addition, we have titrated pools of 10 cervical and 10 lumbar enlargements of the cord; also 10 pairs of olfactory bulbs, all from intranasally infected monkeys, sacrificed within one day after the onset of complete paralysis. The maximum dilution in which the pool of cervical enlargements of the cord infected was 1:8,000; that of the pool of lumbar enlargements was 1:16,000, while the pool of olfactory bulbs infected in a maximum dilution of 1:5,000.

Although the titration figures given above probably do not represent exact endpoints, they give some information as to the approximate concentrations reached in the localities mentioned.

Our observations support the evidence obtained by previous investigators that the virus spreads axonally. In addition, they throw some light on the concentrations attained by the virus during the preparalytic period in those regions through which it passes enroute to the cord and where neuronal damage is much less evident than in the cord.[6] They seem to show, furthermore, that even in the cord the

[5] Sabin, A. B., and Olitsky, P. K., *J. A. M. A.*, 1937, **21**, 108.

[6] Hurst, E. W., *J. Path. and Bact.*, 1929, **32**, 457.

virus may reach rather high concentrations before the usual extensive neuronal damage appears and frank paralysis sets in. The highest concentration seems to be reached at the time of paralysis, which is presumably immediately after the bulk of the anterior horn cells have suddenly crumbled under the action of a certain rather high critical concentration of virus.

Superficially at least, the relationships seem to be analogous to those observed in bacteriophagy in that fairly large amounts of virus may be formed in the absence of apparent nerve cell damage and in that actual cell destruction may be a function of the concentration reached by the virus, as well as of the general level of susceptibility of the cells exposed to it. In other words, virus production and nerve cell damage do not altogether parallel each other. It may be that the amount of damage sustained by the nerve cells in any given region of the nervous system depends rather more on the concentration which a given virus is capable of building up than upon its invasiveness as such.

In the case of bacteriophages it is known that individual bacteriophages on serial passage eventually reach a certain concentration endpoint, either low or high, beyond which they cannot be increased. To us it seems possible that like some strains of bacteriophage, which despite serial passage remain of low titer and low lytic power, there are constantly low titer strains of poliomyelitis virus, which although not lacking in invasiveness may be unable to build up a concentration high enough to damage even the more susceptible nerve cells. Naturally acquired immunity may rest largely on exposure to such low titer strains, which may be quite distinct from those capable of inducing epidemics of the paralytic disease.

10499

Micro Determination of Serum Proteins by Gasometric Carbon Analysis.

CHARLES L. HOAGLAND AND D. JEAN FISCHER. (Introduced by David P. Barr.)

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis.

The successful adaptation of the Van Slyke manometric apparatus to the measurement of carbon in organic compounds by wet combustion is well illustrated by the micro-gasometric measurement of

serum lipids.[1] The small quantity of material needed for analysis, the precision afforded by manometric technic, rapidity and ease in preparation of substances for lipid carbon analysis, led us to attempt the estimation of serum proteins by a technic designed to measure the protein carbon rather than the protein nitrogen which forms the basis for quantitative methods heretofore proposed.

There are several objections to the use of nitrogen data alone for the quantitative expression of protein. First, the commonly accepted use of the factor 6.25 x protein nitrogen = grams of protein takes for granted a constancy in the amino-acid structure of proteins which we have no valid right to assume, particularly in the analysis of abnormal changes in serum proteins occurring in pathological conditions. Di-amino carboxyllic acids and certain of the hetero-cyclic amino-acids, for instance, contain on an average twice as much nitrogen as mono-amino-acids, yet no cognizance is taken of this fact or of the posibility of change in ratio of certain of these amino-acids in pathological states when nitrogen alone is used as the element to which the empirical factor 6.25 is applied for protein conversion. The objection that the use of carbon is also an empirical approximation is met by the fact that while the nitrogen may be increased by as much as 100%, the elementary carbon in di-amino-acids is approximately the same as in the mono-amino group. When multiplied by the nitrogen conversion factor, moreover, such differences in amino nitrogen content may conceivably produce errors of considerable magnitude.

Another argument in favor of carbon as the reference element in the determination of protein is the fact that it forms relatively a much greater percentage of the total protein components than does nitrogen, hence a small error in analytical data is kept relatively less due to the smaller conversion factor.

Another legitimate objection to nitrogen analyses is the well-known difficulty encountered in digestion. The relative ease and simplicity with which carbon combustions may be made obviate many of the difficulties inherent in protein digestion technics preparatory to nitrogen analyses. Moreover, no added determination and correction for non-protein nitrogen is necessary. Last, but perhaps first in importance, the accurate carbon analyses possible on comparatively minute amounts of materials make the carbon combustion technic for protein determination applicable to relatively small amounts of serum (0.1 cc) such as may be obtained readily from finger blood and from small experimental animals.

[1] Van Slyke, D. D., Page, I. H., and Kirk, E., *J. Biol. Chem.*, 1933, **102**, 635.

Technic. Except for the initial dilution, the procedure for a complete analysis can be carried out in one combustion tube, thus obviating the chance of error through losses of material in transference. As many as 20 or more samples may be prepared for analysis at the same time, the limitation being only convenience in handling, centrifuging, and storing of materials preparatory to combustion.

Fractionation is carried out by the Campbell and Hanna[2] method, the filtrate saved for tungstic acid precipitation of remaining protein. The choice of tungstic acid as the protein precipitating agent seemed advisable for several reasons. Folin and Wu[3] early called attention to the fact that non-protein materials were not carried down to any significant degree with tungstic acid, hence the wide adoption of tungstic acid filtrates for differential analyses of non-protein constituents of blood. Moreover, tungstic acid produces a heavy protein aggregation which settles promptly under centrifugation and remains tightly packed through the procedure of capillary suction and removal of supernatant liquid.

Combustion technic and apparatus has been described in detail by Van Slyke, Page and Kirk[1] so that description need not be repeated here. The method of combustion, once fractionation and precipitation have been carried out follows without deviation that proposed by these authors for lipid carbon determination.

Reagents. (All materials are Reagent Quality).

1. Tungstic acid precipitation reagent. Prepared according to the method of Folin, and modified by Haden.[4]
2. Alcohol-ether mixture, 3:1.
3. Sodium sulfite 21%, for use in globulin separation as proposed by Campbell and Hanna.[2]
4. Chromic acid digestion mixture, prepared according to Van Slyke, Page and Kirk.[1]

Procedure. Total Protein Carbon: 0.1 cc of serum is measured carefully into a 10 cc volumetric flask, rinsed from pipette and made to volume with physiological saline. Invert several times to insure thorough mixing. One cc aliquots are measured into pyrex combustion tubes* and the protein precipitated by the addition of 5 cc of tungstic acid reagent. Allow to stand 2-3 minutes for aggregation to occur. Centrifuge 10 minutes at 2000 R.P.M., after which time the

---

2 Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, 1937, **119**, 15.

3 Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

4 Haden, R. L., *J. Biol. Chem.*, 1923, **56**, 469.

* Combustion apparatus may be purchased from Eimer and Amend, New York, Catalogue No. 18237.

protein will be packed tightly at the bottom of the combustion tube. Remove clear supernatant liquid by a capillary tube attached to suction. The precipitate is then washed twice with 5 cc portions of tungstic acid reagent, centrifuging and removing supernatant as above. Finally the precipitate is washed once with 5 cc portions of alcohol-ether mixture to remove any trace of serum lipids which may have been carried down with the precipitate, the supernatant removed with capillary suction. The precipitate is then heated on water bath to drive off traces of alcohol and ether, and set aside under cover for combustion.

Globulin Fractionation: 2.4 cc of 21% sodium sulfite is added to 0.1 cc of serum, the two are mixed several times by gentle inversion and allowed to stand 10 minutes for globulin precipitation to occur. Mixture is then poured twice through the same filter (No. 40 Whatman's) and 0.5 cc aliquots measured into pyrex combustion tubes as described above. The protein is precipitated by 5 cc of tungstic acid reagent plus 2 drops of 10 normal sulfuric acid to neutralize the sodium sulfite present. Allow material to stand for 5 minutes, centrifuge and wash in manner described above for total protein.

Combustion is carried out after the method for organic carbon described by Van Slyke, Page and Kirk,[1] and manometric values converted to milligrams of carbon according to factors given by these authors.

In order to express protein carbon data in terms which could be compared critically with data derived from protein nitrogen determination it became necessary to make simultaneous determination of protein nitrogen with protein carbon. Protein nitrogen was determined by gasometric micro-kjeldahl technic according to the method of Van Slyke.[5] In a series of normal pooled sera, the ratio of carbon to nitrogen was found to be a constant (Table I). This ratio for normal serum protein agrees favorably with previously reported values from elementary analyses.[6]

TABLE I.
Carbon and Nitrogen Analyses of Pooled Serum Protein.

Pooled Sample No. *	Carbon %	Nitrogen %	C/N	N Factor	N Factor / C/N Ratio
A	35.60	10.46	3.40	6.25	1.84
B	34.57	10.09	3.42	6.25	1.83
C	30.05	8.86	3.395	6.25	1.84

*Each pooled sample contained 10 normal sera.

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1926, **71**, 235.

[6] *Handbuch der Biochemie des Menschen und der Tiere*, 1909, pp. 280-285.

TABLE II.
Total Protein by Carbon Analysis.

Sample No.	Mg Carbon/cc	Carbon Factor	Total Protein, g %
1	.300	1.84	5.52
	.301	1.84	5.53
2	.3645	1.84	6.71
	.364	1.84	6.70
3	.3555	1.84	6.54
	.356	1.84	6.55
4	.379	1.84	6.98
	.378	1.84	6.96

Albumin and Globulin by Carbon Analysis

Sample No.	Total Carbon mg/cc	Albumin Carbon mg/cc	g %		
			Total Prot.	Albumin	Globulin*
1	.300	.1775	5.52	3.26	2.26
	.301	.1765	5.53	3.25	2.28
2	.314	.1875	5.78	3.45	2.33
	.317	.1865	5.83	3.43	2.40
3	.348	.2010	6.41	3.70	2.71
	.349	.2000	6.43	3.68	2.75
4	.361	.2102	6.64	3.87	2.77
	.3615	.2110	6.65	3.88	2.77

*By difference.

In Table II are listed duplicate pairs of total protein and globulin-by-difference values, indicating the degree of consistency possible by the protein carbon technic. In all instances the second decimal place is significant.

The carbon ratios given above represent determinations on normal sera. An investigation into the nitrogen and carbon content of serum proteins occurring in various pathological conditions is under way. Also an attempt is being made to apply carbon combustion technic to the determination of other serum and plasma constituents, such as fibrinogen, euglobulin, pseudoglobulin, etc. It is felt that carbon analyses, in addition to nitrogen studies, may help to characterize certain pathological proteins more closely than is now possible with nitrogen analyses alone.

Summary. 1. A manometric micro-method for the determination of serum proteins by protein carbon estimation has been outlined which gives consistently reproducible results on as little as 0.1 cc of serum. 2. Certain advantages, both technical and theoretical, in the use of carbon rather than nitrogen as the reference element in serum protein determination is discussed.

10500 P

Carbohydrates of Certain Vegetables and Fruits.

WILLIAM H. OLMSTED AND RAY DAVID WILLIAMS.

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis.

A method is presented for the direct determination of the carbohydrate fraction of several classes of foods. The principal features of the method are: Materials very low in carbohydrate are concentrated by air-drying below 60°C until the residue can be pulverized. The soluble sugars are separated from the starch by solution in 60% ethyl alcohol. After inversion of the sucrose the sugars are determined before and after fermentation. The starch is separated from the unavailable carbohydrate residue by pancreatin digestion and further acid hydrolysis of the filtrate to convert the starch into glucose which is determined as such. The residue is then dissolved in 21.4 N sulfuric acid and further hydrolyzed to convert the cellulose into glucose, the hemicellulose into non-fermentable sugars. The lignin which is insoluble in dilute acid is determined gravimetrically.

Data obtained by this method and certain conventional procedures in a composite analysis account for more than 98% of the carbohydrate in a variety of common foods. Our results compare favorably in regard to available carbohydrates with those obtained by reliable direct determinations in other laboratories. We know of no data with which to compare our results on the unavailable carbohydrate fraction. In a previous publication[1] we emphasized the errors of the old crude fiber determinations.

10501 P

Aminoethyl Phosphoric Ester in the Small Intestine of Rabbits and Pigs.

SIDNEY P. COLOWICK AND CARL F. CORI.

From the Department of Pharmacology, Washington University School of Medicine, St. Louis.

Outhouse reported the isolation of this ester from bovine malignant tumors, but found no trace of it in benign tumors or in the normal tissues investigated which included muscle, placenta, pancreas, liver

[1] Williams, R. D., and Olmsted, W. H., *J. Biol. Chem.*, 1935, **108**, 653.

and embryo.[1, 2] Consequently, he expressed the belief that this ester is peculiar to malignant tissue.

In a study of the acid-soluble phosphoric esters of the intestine in which a method of fractionation similar to that of Outhouse was used, small amounts of a crystalline quinine salt were isolated from both rabbit and pig intestine. This quinine salt dissociated when boiled in ethyl alcohol, leaving a crystalline residue which after several recrystallizations from 80 to 90% ethyl alcohol showed a N and P content close to the theory for aminoethyl phosphoric ester. A sample of synthetic ester, prepared in this laboratory by Dr. Welch according to the method of Plimmer and Burch,[3] was in all respects identical with the ester isolated from the intestine.

A much better yield of this substance was obtained by means of a new method involving fractionation of the phosphoric esters as the uranium salts. This method is based on the fact that uranium acetate precipitates aminoethyl phosphoric ester, nucleotides and other esters quantitatively from a neutralized trichloracetic acid extract of intestine, but forms soluble salts with compounds which contain no phosphorus. All other salts of aminoethyl phosphoric ester, including the basic lead salt, were found to be water-soluble. By shaking the uranium salts with a solution of $Ba(OH)_2$ the aminoethyl phosphoric ester is extracted as the Ba salt, leaving most of the other esters in the precipitate. After a second precipitation with uranium, extraction with $Ba(OH)_2$, concentration of the barium-free solution, and removal of a small precipitate formed on addition of mercuric acetate, the ester can be crystallized directly from 90% alcohol. After several recrystallizations and drying of the substance at 110°, a sample showed 10.2% N and 21.8% P; calc. for $C_2H_8O_4NP$; N 9.9%, P 22.0%.

In a preparation in which 137 g of rabbit intestine were used the yield of the ester per 100 g wet weight was 40 mg, which is about the same as the amount found in malignant tumors by Outhouse. It is estimated that the actual content in the small intestine is about twice as high, so that this ester would account for about ¼ of the acid-soluble organic P.

The barium hydroxide insoluble uranium fraction contains large amounts of a nucleotide which has been isolated in crystalline form and identified as adenylic acid; it corresponds roughly to ¼ of the acid-soluble organic P. The adenylic acid may not occur as such in the

1 Outhouse, E. L., *Biochem. J.*, 1936, **30**, 197.

2 Outhouse, E. L., *Biochem. J.*, 1937, **31**, 1459.

3 Plimmer, R. H. A., and Burch, W. J. N., *Biochem. J.*, 1937, **31**, 398.

intestine, but may be formed during the course of the preparation from some more complex nucleotide. A third fraction corresponding to 12 to 16 mg P per 100 g intestine is not precipitated by uranium. The nature of the phosphate esters in this fraction has not been established.

Summary. A fractionation of the acid-soluble organic phosphoric esters of the small intestine as the uranium salts has been described. Aminoethyl phosphoric ester and adenylic acid have been isolated in crystalline form, each comprising about ¼ of the acid-soluble organic P of the rabbit small intestine. The occurrence of the former ester in pig intestine has also been demonstrated.

10502

Effects of Caffeine on Human Sugar-Tolerance Curves.*

MARTIN DEAKINS. (Introduced by H. C. Hodge.)

From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York.

There is considerable evidence that caffeine affects the blood sugar of animals. In dogs, a rise follows caffeine injection[1] amounting in some cases to 40-60% above normal[2] in both arterial and venous blood;[3] a double dose is necessary to produce hyperglycemia during avitaminosis.[4] During work,[5] the sugar tolerance curve rises more rapidly with caffeine than without and stays up longer.

However, caffeine produces no hyperglycemia in castrated female rabbits,[6] nor does it affect the glycogen contents of the heart or liver in rats and guinea pigs.[7]

No report could be found regarding the effect of caffeine on the blood sugar or sugar tolerance curve for man.

Eight sugar tolerance curves were determined on the same subject (M.D.) using the sugar method of Folin and Malmros.[8] The nutri-

* This work was financed by a grant from the Carnegie Corporation of New York.

1 Karger, K., *Klin. Wochschr.*, 1927, **6**, 1994.

2 Labbe, H., and Theorodesco, B., *Compt. rend.*, 1924, **178**, 886.

3 Bömer, M., *Arch. exp. Path. Pharmakol.*, 1930, **149**, 247.

4 Collazo, J. A., and Gohse, S. N., *Biochem. Z.*, 1923, **139**, 285.

5 Atzler, E., *Arbeitsphysiol.*, 1938, **10**, 30.

6 Laufberger, W., *Z. ges. exp. Med.*, 1924, **39**, 487.

7 Laseh, F., and Triger, K., *Z. ges. exp. Med.*, 1933, **88**, 588.

8 Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

tional history was nearly the same in all cases. No food was taken after 5 p. m. the day before, the amount of sleep and activity was similar, and the determinations were made at the same time of day, 10 a. m.—1 p. m. Fingertip blood samples were taken every 10 minutes for the first 1½ hours after ingesting the dextrose. All sugar determinations were made in duplicate.

The data and results from 4 of the experiments are shown in Fig. 1.

These results indicate that large doses of caffeine depress the peak of the human sugar-tolerance curve and slightly delay the return to normal. Caffeine alone does not raise the blood sugar appreciably. The amounts commonly ingested in coffee and tea probably do not significantly affect either the human tolerance curve or the blood sugar levels.

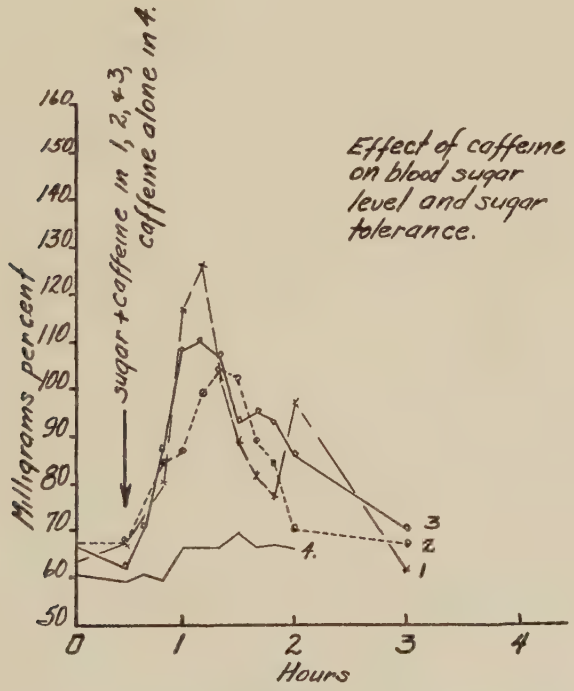


FIG. 1.

1. 50 g dextrose + 200 cc distilled water.
2. 50 g dextrose + 0.5 g caffeine + 200 cc distilled water.
3. 50 g dextrose + 200 cc coffee (9 g coffee/100 cc water).
4. 0.5 g caffeine + 200 cc distilled water.

10503

Relative Effectiveness of Ingested and Injected Manganese in Preventing Perosis.*

C. D. Caskey† and L. C. Norris.

From the Department of Poultry Husbandry, Cornell University, Ithaca, New York.

Manganese was shown by Wilgus, Norris and Heuser[1] to be markedly effective in preventing the development of the osteodystrophy of chicks, named perosis by Titus and Ginn.[2] Prior to this discovery it was found by Hunter, *et al.*,[3, 4] and others that diets high in calcium and phosphorus are in general more perosis-producing than diets containing less of these constituents. Gallup and Norris[5] reported that extremely high levels of manganese in high calcium-phosphorus diets are ineffective in preventing a small residual amount of perosis in Rhode Island Red and New Hampshire chicks. They[6] also reported that under such a dietary regime little manganese is retained in the body of the chick.

In view of these results a study was undertaken to determine if the need for dietary manganese is increased by an increase in the calcium and phosphorus content of the diet. A further purpose was to determine if the causal relation between high calcium-phosphorus diets and perosis is due to the effect of an excess amount of these mineral elements upon the availability of manganese in the intestinal tract.

Rhode Island Red chicks were used as the experimental subjects in this study. They were quartered in battery brooders with wire-mesh floors so as to reduce coprophagy as much as possible. Water was supplied in non-metallic drinking vessels. The experimental period was of 6 weeks' duration.

The basal diet was composed of 40% ground yellow corn, 25.25%

* This work was made possible by the establishment of a Special Temporary Fellowship at Cornell University, by the Grange League Federation Exchange, Inc., of Ithaca, New York.

† On leave of absence from the Oklahoma Agricultural and Mechanical College, 1937-39.

1 Wilgus, H. S., Norris, L. C., and Heuser, G. F., *J. Nutr.*, 1937, **14**, 155.

2 Titus, H. W., and Ginn, W. M., *Science*, 1931, **74**, 249.

3 Hunter, J. E., and Funk, E. M., *Proc. Poul. Sci. Assn.*, 1930, 22nd ann. mtg., 45.

4 Hunter, J. E., Dutcher, R. A., and Knandel, H. C., *Poul. Sci.*, 1931, **10**, 392.

5 Gallup, W. D., and Norris, L. C., *Poul. Sci.*, 1939, **18**, 76.

6 Gallup, W. D., and Norris, L. C., *Poul. Sci.*, 1939, **18**, 99.

degerminated yellow corn meal, 15% dried skim milk, 5% dehydrated alfalfa meal, 8% commercial casein, 3% meat scrap, 2% liver meal, 1% calcium carbonate, 0.5% iodized salt and 0.25% cod liver oil, containing 250 U.S.P. units of vitamin D per gram.

This diet contained approximately 20% protein, 1% calcium, and 0.5% phosphorus and was designated the low calcium-phosphorus diet. By substituting 7% of bone meal for an equal amount of ground yellow corn in this diet a high calcium-phosphorus diet, containing approximately 3% calcium and 1.5% phosphorus, was obtained.

Manganese was supplied both by incorporation in these diets and by intraperitoneal injection. In the diets c.p. manganous carbonate was used as the source of manganese and in preparing the solutions for injection c.p. manganous chloride was used. A sufficient quantity of sodium chloride was added to each solution so as to make it isotonic with blood.

All the injections were made at 3-day intervals starting when the chicks were 3 days of age. The quantity of solution injected the first time was 0.5 cc. Thereafter it was increased at every second injection by 0.5 cc. A total of 28 cc was injected during the experimental period.

Four lots of 25 chicks each were fed the low calcium-phosphorus diet, supplemented with sufficient manganese to give levels of 0.5 mg, 1.5 mg, 2.5 mg, and 3.5 mg per 100 g of diet respectively. Four lots of chicks were fed the high calcium-phosphorus diet, supplemented with 0.5 mg, 3.5 mg, 7 mg and 14 mg per 100 g of diet respectively. Three lots of 10 chicks each were fed the high calcium-phosphorus diet accompanied by injection of 10 mg, 20 mg, and 60 mg of manganese respectively as previously indicated.

The results of supplying manganese in the diet (Table I) show that, as the manganese level in either the low calcium-phosphorus diet or the high calcium-phosphorus diet was increased, the final average weight of the chicks increased and the incidence of perosis decreased. The apparent exception in the high calcium-phosphorus lot receiving 0.5 mg of manganese per 100 g of diet was due probably to the severe retardation of growth in this lot which according to Gallup and Norris[7] lowers the incidence of perosis. The results also show that a level of 1.5 mg of manganese per 100 g of a diet containing 1% of calcium and 0.5% of phosphorus was just as effective in preventing the development of perosis as a level of 14 mg of manganese per 100 g of a diet containing 3% of calcium and 1.5%

[7] Gallup, W. D., and Norris, L. C., *Poul. Sci.*, 1937, **16**, 351.

TABLE I.
Relationship Between Level of Dietary Calcium and Phosphorus and Level of Dietary Manganese Needed to Prevent Perosis.

Exp.	Chicks per lot	Mg Mn. per 100 g diet	Mg Mn. ingested in 6 wks	Avg wt in g at 6 wks	% of perosis	Severity[1] index
		Calcium 1.0%, phosphorus 0.5%.				
1	25	0.5	4.4	410	61	26.4
	25	1.5	15.5	500	27	10.7
	25	2.5	26.9	515	0	0
	25	3.5	38.0	525	5	2.7
		Calcium 3.0%, phosphorus 1.5%.				
1	25	0.5	3.6	270	42.0	20.5
	25	3.5	28.6	310	66.6	33.9
	25	7.0	60.9	400	44.4	16.6
	25	14.0	141.7	500	26.0	8.0

[1] See reference 1.

of phosphorus. Hence, it is evident that high levels of calcium and phosphorus by increasing the need for dietary manganese interfere in some manner with the effectiveness of this mineral element.

The results of supplying manganese by intraperitoneal injection (Table II), show that 10 mg of manganese per chick during the experimental period completely prevented the development of perosis in spite of the fact that the chicks received a diet abnormally high in calcium and phosphorus whereas the oral intake of 141.7 mg of manganese was only partially effective. The injection of 20 mg and 60 mg of manganese during the experimental period retarded growth considerably. In previous work Gallup and Norris[5] found that the oral intake of approximately 1100 mg of manganese per chick during the same period of time by means of a diet containing 100 mg of manganese per 100 g had no detrimental effect upon growth.

The fact that small amounts of manganese when injected intraperitoneally are completely effective in preventing the development of perosis in chicks fed a high calcium-phosphorus diet was confirmed by the results of a second experiment. In this experiment a lot of chicks injected with an isotonic saline solution containing no manganese served as a control.

From these results it is evident that calcium and phosphorus when present in the diet in excess amounts greatly reduces the availability of manganese in the intestinal tract. This conclusion is supported by the work of Becker and McCollum,[8] who found that diets high in soluble manganese are not toxic to rats provided sufficient phosphorus is present. It is also supported by the work of Blumberg,

[8] Becker, J. E., and McCollum, E. V., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 740.

TABLE II.
Effect of Intraperitoneal Injection of Manganese in Preventing Perosis.

Exp.	Chicks per lot	Injection treatment	Mg Mn. injected in 6 wks	Avg wt in g at 6 wks	% of perosis	Severity index
		Calcium 3%, phosphorus 1.5%.				
1	25	None	0	270	42	20.5
	10	Isotonic $MnCl_2$-NaCl soln	10	444	0	0
	10	" $MnCl_2$-NaCl "	20	357	0	0
	10	" $MnCl_2$-NaCl "	60	277	0	0
		Calcium 2.5%, phosphorus 1.25%				
2	20	Isotonic NaCl soln	0	320	40	14
	20	" $MnCl_2$-NaCl soln	14	360	0	0

Shelling and Jackson,[9] who obtained rickets in rats by including large amounts of either manganous chloride or manganous carbonate in diets in which the calcium-phosphorus ratio was 1.1 to 1. Still further support is provided by the results of Wilgus and Patton,[10] who found that the amount of dializable manganese in the intestinal tract of chicks decreases with an increase in the calcium and phosphorus content of the diet.

Summary. The relative effectiveness of ingested and injected manganese in preventing the development of perosis in chicks fed a high calcium-phosphorus diet was studied. The results obtained showed that excess calcium and phosphorus in the diet greatly reduces the availability of manganese in the intestinal tract.

10504 P

Experimental Production of Leprosy in the Rabbit with Chrome Acid-Fast Culture.

J. R. KRIZ AND W. L. LOVING. (Introduced by C. W. Duval.)

From the Department of Pathology and Bacteriology, School of Medicine, Tulane University, New Orleans.

It has been noted[1] that the rabbit is susceptible to inoculations with culture of Duval's chromogenic acid-fast bacillus from leprosy. Observations on its behavior to infection by an old and recent chrome acid-fast culture from the human leprous lesion are, therefore, described here. *B. smegmatis* and *B. phlei* were used as controls.

9 Blumberg, H., Shelling, D. H., and Jackson, D. A., *J. Nutr.*, 1938, **16**, 317.
10 Patton, A. R., and Wilgus, H. S., Jr., *J. Nutr.*, 1939, in press.
1 Kriz, J. R., *Am. J. Trop. Med.*, 1938, **18**, 213.

Twenty-four full grown healthy rabbits were divided into 4 groups of 6 each. The various groups received 2 cc of a heavy suspension of the respective cultures. Subcutaneous and intraperitoneal injections were made at weekly intervals for a period of 3 weeks. Four months following the last injection, 2 animals from each group were sacrificed and observed for gross and microscopic lesions. At time of autopsy blood cultures were made and material from the animal lesions were inoculated on various special media in an attempt to recover the microörganism used as the inoculant. The records of the observations made on the first series, *i. e.*, 4 months after the last injection, are summarized in Table I. Histological sections of all organs were stained with hematoxylin-eosin and also prepared by the Ziehl-Neelsen method.

The results of the first series of experiments show that there are significant differences among the 4 groups. It is interesting to note that the animals in Group 2, inoculated with the recent culture, produced the most marked changes, indicating a greater virulence for the more recent isolation. The gross lesions seen in animals of Group 2 resemble closely those found in human leprosy while the microscopic changes were identical to the human lesion, namely, aggregations of lymphoid and epithelioid cells with many mononuclear "foamy" cells containing myriads of acid-fast bacilli, so-called "globi."

The chromogenic acid-fast bacillus of leprosy employed in the experiments was cultured from the gross lesions only after incubation

TABLE I.

	Gross Lesions	Microscopic	Culture
Group 1. *B. lepræ* (old culture)			
Rabbit A	Few pin-point nodules in liver.	Epithelioid, lymphoid, occasional giant cell.	+
" B	None	None	—
Group 2. *B. lepræ* (recent culture)			
Rabbit A	Many large discrete nodules in liver, spleen, omentum, mesentery	Epithelioid, lymphoid, giant cells and "globi" with acid-fast bacilli.	+
" B	"	"	+
Group 3. *B. smegmatis*			
Rabbit A	None	None	—
" B	"	"	—
Group 4. *B. phlei*			
Rabbit A	None	None	—
" B	"	"	—

at 37.5°C for 26 days. Morphologically and tinctorially the recovered culture was identical to the organism used as the inoculant. Likewise a chromogenic acid-fast bacillus was cultured from the liver of the rabbit from Group 1, requiring 18 days before visible growth was noted. Cultures were not recovered from the control animals of Groups 3 and 4. Although Duval and Harris[2] pointed out that saprophytic acid-fast microörganisms produce lesions in the experimental animal following repeated massive doses 4 to 6 weeks after the last injection, no lesions were noted in any of our control animals, 4 months after the last injection.

Summary. The recent chrome acid-fast culture produces lesions in the rabbit that grossly and microscopically resemble those seen in human leprosy. Here, the lesion is definitely progressive over a period of 4 or more months. During this period it is noteworthy that the exciting microörganism steadily increases in number. The "foamy" cells are crowded with dense colony-like masses of the specific bacillus. The chromogenic acid-fast bacillus was recovered in culture from the lesions of the animals 4 months following the last injection. The isolation of the bacillus used in the experiment was more difficult from the lesions that were 4 months old than from the earlier lesions. Visible growth occurred only after 4 weeks' incubation. In the control animals no gross or microscopic lesions were noted 4 months after the last inoculation. The cultures inoculated with material from these animals remained sterile after 4 weeks' incubation.

10505 P

Control of Vitamin K Therapy. Compensatory Mechanisms at Low Prothrombin Levels.*

S. E. ZIFFREN, C. A. OWEN, G. R. HOFFMAN AND H. P. SMITH.

From the Department of Pathology, State University of Iowa, Iowa City.

The use of vitamin K to combat the lowered plasma prothrombin level in jaundiced bleeders[1, 2] has created a demand for a simple

2 Duval, C. W., and Harris, W. H., *J. Med. Res.*, 1913, **23**, 165.

* Aided by a grant from the John and Mary R. Markle Foundation. Funds for two research assistants were supplied by the Graduate College, State University of Iowa.

1 Warner, E. D., Brinkhous, K. M., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 628; *Am. J. Med. Sci.*, 1938, **196**, 50.

2 Butt, H. R., Snell, A. M., and Osterberg, A. E., *Proc. Staff Meetings of the Mayo Clinic*, 1938, **13**, 65; *ibid.*, 1938, **13**, 753.

clotting test. Quick[3] has suggested the use of whole blood as a test for thromboplastic activity; we propose to reverse the procedure, and use thromboplastin, in large amounts, to test the clotting power of whole blood. We also propose to show that deficiency in prothrombin can be compensated in part by changes in other factors.

The test. In a 3 cc tube is placed 0.1 cc thromboplastin. Blood, freshly drawn, is run into the tube to a 1.0 cc mark, then inverted once, and tilted gently every few seconds, and the clotting time observed. The test is repeated on a normal individual, and the unknown expressed in percentage of the normal.

$$\text{Clotting activity (in \% of normal)} = \frac{\text{Clotting time of the normal}}{\text{Clotting time of the unknown}} \times 100$$

For example, a patient's blood clotted in 60 seconds, a control in 30. Activity, therefore, was 50% of normal.

To prepare thromboplastin, extract 10 g ground brain or lung (ox or rabbit) 2 hours with 10 cc saline; strain, and preserve in ice box. Variable potency does not affect the clotting time ratio of unknown to control. If normal values exceed 60 seconds, the thromboplastin is rejected; if less than 25 seconds one should dilute the thromboplastin with saline.

Results. Table I shows results on 10 cases selected at random from our series. The prothrombin levels obtained by the 2-stage titration procedure developed in this laboratory[4] serve as a standard. It is seen that the values obtained with the new test usually agree within 15% with the true prothrombin levels, indicating that the new test is dependent in large part upon the prothrombin concentration.

When prothrombin falls below the level of 30-50% a bleeding tendency appears. Above this level blood clots at a normal rate (6-10

TABLE I

Diagnosis	Sex	New test (in % of normal)	Quantitative prothrombin test (in % of normal)
Pernicious Anemia	F	100	98
Aplastic "	M	93	83
Cholecystitis	F	90	92
Obstr. Jaundice	M	76	80
" "	M	62	73
" "	M	60	60
Biliary Fistula	F	50	49
Obstr. Jaundice	M	41	46
"Toxic Hepatitis"	M	31	23
Obstr. Jaundice	M	22	13

[3] Quick, A. J., *Am. J. Physiol.*, 1936, **114**, 282.

[4] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667; *J. Exp. Med.*, 1937, **66**, 801.

min.), suggesting, we believe, that other factors vary to compensate for any prothrombin deficiency which may exist. In one case, not listed in the table, the new test consistently gave normal values, whereas the 2-stage titration showed that the prothrombin was, in reality, 50% of normal. Since thromboplastin is eliminated as a variable by the test, it follows that some other factor varied, permitting thrombin to be formed with normal speed[5] despite a deficiency in prothrombin. In most cases, however, the new test, by eliminating thromboplastin variations, gives values corresponding to the true prothrombin level. This suggests that thromboplastin itself may vary in amount, effecting compensation.

The new test, like the "prothrombin test" of Quick,[3] measures not prothrombin alone, but the summation of several variables, and it thus supplies a practical measure of the tendency to bleed. It is a simplification of his test, in that thromboplastin is added directly to whole blood instead of to plasma. This eliminates centrifugalization, recalcification, and titration. It is a bedside test which we have found to be useful as a guide for vitamin K therapy.

10506 P

Effect of Riboflavin-low Diets upon Nerves, Growth, and Reproduction in the Rat.*

R. W. ENGEL AND P. H. PHILLIPS. (Introduced by E. B. Hart.)

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

While investigating the nervous system of growing chicks maintained on a riboflavin-low diet (Phillips and Engel[1, 2]) it was discovered that severe degeneration occurred in the myelin sheaths of the spinal cords and the peripheral nerves. The purpose of this study was to determine whether similar nervous system changes would occur in the rat raised under similar dietary conditions.

5 Warner, E. D., Brinkhous, K. M., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 197.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were aided in part by the Works Progress Administration. Supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 Phillips, P. H., and Engel, R. W., *Poul. Sci.*, 1938, **17**, 463.

2 Phillips, P. H., and Engel, R. W., *J. Nutr.*, 1938, **16**, 451.

The basal ration used in the chick studies consisted of:

Yellow corn	59	NaCl	1
EtOH extracted casein	12	$Ca_3(PO_4)_2$	1
Wheat bran	5	$CaCO_3$	1
Whole wheat	20	Cod liver oil	1

The riboflavin content of this ration was further reduced in this study by replacing 40% of the yellow corn with dextrin and by using a purified casein washed and reprecipitated 4 times.

Two normal adult female albino rats were maintained on this ration for a period of 5 months, one of them receiving a supplement of 25 mg of nicotinic acid per kilo of ration. During this experimental period each female bore and reared 3 litters of young. The mothers maintained their original weight over the 5-month period and no deficiency symptoms became apparent. The pups were normal in size and appearance, and numbered from 6 to 11 per litter. Twelve pups were autopsied during the suckling period; 16 were weaned at 3 weeks of age and fed the deficient ration which their mothers were receiving. Twelve of these were removed for histologic study between 3 and 5 weeks of age. The remaining 4 were fed the basal diet and allowed to grow to maturity. Two of these were given the basal ration plus nicotinic acid at the rate of 25 mg per kg. These 4 animals grew at the rate of only 12 g per week but they did not develop nervous symptoms. This would indicate that the ration was sufficient in riboflavin but lacking a growth factor or factors for the rat, and that nicotinic acid did not improve the ration.

A total of 24 animals were thus autopsied at ages ranging from 1 day to 5 weeks. Routine examination of the spinal cord and peripheral nerves by methods previously described (Engel and Phillips[3]) revealed normal nerve structure. Brain, liver, and kidney, stained with H and E were examined and found to be normal except for occasional cases of vacuolization in the liver parenchyma.

Summary and Conclusions. 1. The severe nerve degeneration characteristic of growing chicks fed a riboflavin-low ration failed to occur in rats maintained on a similar ration. 2. A ration low in riboflavin for the chick had no adverse effect upon reproduction in the rat. 3. This ration apparently lacks a growth-promoting factor for the rat. The addition of nicotinic acid did not improve the ration.

[3] Engel, R. W., and Phillips, P. H., *J. Nutr.*, 1938, **16**, 585.

10507

Crown Gall-Like Tumors Induced with Scharlach Red on the Plant, Kalanchoe.

MICHAEL LEVINE.

From the Laboratory Division, Montefiore Hospital, New York.

The reaction of plants to carcinogenic agents, growth substances, vitamines, and heteroauxins has been intensively studied by a number of workers during the past few years. Primarily, their purpose has been to determine the physiological responses made by various garden and house plants to these chemical substances. Other botanists have been interested in determining whether these chemical agents were capable of producing overgrowths analogous to crown gall, induced by *Pseudomonas tumefaciens,* which has been considered cancer[1] of plants, and which they contended were similar to cancer in animals and man.

In earlier reports,[2, 3, 4] the writer has shown that a large number of chemical substances produced small proliferations on susceptible plants but which lacked the essential characteristics of crown galls. In a category of neoplastic diseases the position of these overgrowths was of the lowest order, in that their cell multiplication was limited, differentiation of the induced meristematic activity was rapid and reached its final stage in development before it had developed into a sizable mass. Crown galls on similar plants, grown under similar conditions, and produced by inoculation with a virulent strain of the bacteria, were of a higher grade than the chemical plant "tumors", while cancer in animals and man was the highest order because of its limitless growth and malignancy. Studies on plants made with the heteroauxin, indole acetic acid, have shown that while these plants responded by producing an abundance of roots over the treated areas, swellings also occurred; and where the applications were made on decapitated bean seedlings, the cut surface produced clusters of intumescences which have been described as "tumors".[5, 6, 7]

1 Smith, E. F., Brown, N. A., and Townsend, C. O., *U. S. Dept. Agr. Bull.*, 1911, **213**, 1.

2 Levine, M., *Bull. Torrey Bot. Club*, 1934, **61**, 103.

3 Levine, M., *Bull. Torrey Bot. Club*, 1936, **63**, 177.

4 Levine, M., and Chargaff, E., *Am. J. Bot.*, 1937, **24**, 461.

5 Brown, N. A., and Gardner, F. E., *Phytopathology*, 1936, **26**, 708.

6 Kraus, E. J., Brown, N. A., and Hamner, K. C., *Bot. Gaz.*, 1936, **98**, 370.

7 Link, G. K. K., Wilcox, H. W., and Link, A. DeS., *Bot. Gaz.*, 1937, **98**, 816.

A study[8] of the effects of scharlach red on the *Lycopersicum esculentum,* of the cherry stone variety, showed, on microscopic examination, abundant hyperplastic tissue and some hypertrophy. These suggested the possibility of continued growth under more suitable conditions. In view of the continued favorable results obtained with scharlach red in producing cancer in guinea pigs, rats, and mice reported by Yoshida,[8] Shear,[9] and others, it appeared advisable again to include this dye in further studies on plants. At the same time the 3 principal carcinogenic hydrocarbons, namely, benzpyrene, methylcholanthrene, and 1,2,5,6-dibenzanthracene, were again tested.

During the last 2 years a large number of species of garden and house plants were grown in the open, and under more sheltered conditions. *Kalanchoe Daigremontiana* (Hamet et Perrier) was found to produce crown gall-like overgrowths, with leafy shoots and roots, after a single application of scharlach red suspended in ether. The nature of these masses is so much like those induced by *P. tumefaciens* that it seems desirable to call attention to them now.

The amount of chemicals for each plant was not determined. A paste consisting of 1% to 2% of the chemical thoroughly mixed with lanolin was applied by means of a glass rod. Each plant was treated with scharlach red (0.2 g suspended in 20 cc of purified ether) by a single painting with a camel's hair brush. The controls were on the same or other plants of the same species treated in the same manner but with lanolin (hydrous) only. The procedure consisted of decapitating the growing or apical portion of the plant down to a point somewhat below the middle of the second or third internode. The cut portion of the stem was further injured by pricking it with a sterile, steel needle. More than 50 plants were used in these tests.

Observations. Decapitated stems of Kalanchoe treated with 1% scharlach red in ether produce an abundance of roots after 10 to 12 days. Most of these adventitious roots appear to arise from the node, while the remnant of the severed internode shows slight swelling about the treated surface. The roots increase in number for several days, forming long matted structures. The roots are white and measure more than an inch in length and .25 mm to .5 mm at the base. As the roots grow old they become twisted, reddish brown in color, at the same time, new, white roots make their appearance. At about 25 days after the treatment, the short, internode stub showed a small, smooth, globular mass about the size of a pea above the cluster of roots; this grew rapidly. In the meantime, one of the

[8] Yoshida, T., *Gann,* 1934, **28**, 454.

[9] Shear, M. J., *Am. J. Cancer,* 1937, **29**, 269.

axillary buds alongside the mass developed into an apparently normal shoot.

At approximately 50 days after treatment (Fig. 2), the globular mass developed a number of smooth, confluent bodies with leaf-like projections from the upper surface and roots from its lower part. These galls are faintly green to a yellowish white in color. Streaks of red pigmented tissue are seen in the lower glaucous, under surface of the largest mass. One of these tumors, more than 6 months after treatment is shown on a Kalanchoe in Fig. 1. Here a typical crown gall-like structure is seen with leafy shoots and with an abundance of old and young roots. The lower part of the overgrowth is studded with many protruding, thick, conical structures which appear to be modified root-like organs. These structures are faintly pink in color.

On removing a part of one of the galls for microscopical studies, the macroscopical appearance of the cut surface was indistinguishable from gross sections of crown gall tissue produced in this and other species of plants by the bacteria. The tissue generally is white with deposits of chlorophyll near the abortive, leafy structures; the latter are intensely green. It appears, from the macroscopical structure of these chemically formed galls, that they are identical with those produced by *P. tumefaciens*. The ultimate fate of these scharlach red tumors or their effects on the host are unknown.

Kalanchoes treated with the heteroauxin, indole acetic acid (3% mixture) show essentially different responses as in Fig. 4. The decapitated internode, when covered with a paste of this agent, becomes covered with long, white roots which persist for over a month. In other cases when the internode is covered with the heteroauxin, nodular masses occur with short, stubby roots. These masses necrotize, and at the same time the upper axillary buds develop into normal active shoots. The roots in the former instance wither; they mat themselves about the stem, which frequently cracks, and form only small nodules or intumescences. The tip of the excised internode produces a normal shoot. No tumors that are comparable with crown gall result.

The application of a 2% 1,2,5,6-dibenzanthracene in a lanolin paste to the cut end and surface of the decapitated internode portion of the Kalanchoe, invariably produces an abundance of persistent roots over the treated area. These are thick in structure and white in color. They persist for many months, branch, and become brown, yet young freshly formed white roots appear among them (Fig. 3). Thickened scars have been observed over the treated areas on similar

FIG. 1.
Crown gall-like tumor on Kalanchoe; stem treated with 1% scharlach red on 7-12-1938; photographed 1-20-1939. × ½.

FIG. 2.
Early stage of the same tumor; photographed 8-29-1938. × 1½.

FIG. 3.
Kalanchoe treated with 2% 1,2,5,6-dibenzanthracene in lanolin 7-12-1938; photographed 1-20-1939. This plant shows no tumor, instead roots. × ½.

FIG. 4.
Kalanchoe treated with 3% indole acetic acid in lanolin 8-17-1938; photographed 1-20-1939, shows only roots. × ½.

plants. Normal shoots develop from the edges of the cut surfaces of the internodes. No tumors have been observed on the Kalanchoes so treated.

A 1% paste of either methylcholanthrene or benzpyrene applied to Kalanchoes frequently caused necrosis of the internode remnant, down to the node. However, it is of interest to note that the axillary buds in plants treated with these agents developed into normal shoots. Further, long, white roots, which branched occasionally, appeared at the nodes and internodes below the treated regions. These roots generally formed in a single row on one side of the stem. These agents failed to induce tumors of any sort.

Control studies made on Bryophyllum and Kalanchoes treated with lanolin alone differed in that in the former species no reaction occurred. In the Kalanchoes the treatment with lanolin resulted in the development of a cluster of roots over the treated area. No tumors or nodular masses were observed. It appears from these experiments that the stimulus to root formation on the Kalanchoes treated with the carcinogenic agents is due to factors other than the applied chemicals. It appears evident that the adventitious roots are not the products of tumors. It seems possible that these roots may be the product of the host acted upon by some normal plant substance such as a wound hormone, and that the production of these roots may be independent of bacterial products[7] or of stimuli produced by tumor tissue induced by bacteria.[10]

Conclusions. Scharlach red, dissolved in ether and applied to decapitated shoots of *Kalanchoe Daigremontiana,* produces crown gall-like overgrowths which are characterized by leafy shoots and roots, and which resemble typical crown galls induced by *P. tumefaciens.*

Other carcinogenic agents as 1,2,5,6-dibenzanthracene, methylcholanthrene, and benzpyrene applied in lanolin cause injuries to the treated stem without inducing overgrowths on the Kalanchoe. Roots are produced by the Kalanchoes below and above the areas treated with 1,2,5,6-dibenzanthracene, methylcholanthrene, and benzpyrene. Indole acetic acid induces an abundance of long, white roots on the Kalanchoe, together wth small intumescences which are viable for only short periods. Roots are also produced on injured Kalanchoes when treated with lanolin alone. Root formation on the Kalanchoe induced by substances other than the heteroauxin studied here, results from injury which stimulates the host cells to produce root-forming substances. These substances appear to be transported to parts of the stem below and above the treated areas.

[10] Locke, S. B., Riker, A. J., and Duggar, B. M., *J. Agr. Res.,* 1938, **57**, 21.

10508 P

Vitamin C Requirement of Man. Prolonged Study of Daily Excretion and Plasma Concentration of Vitamin C.*

Elaine P. Ralli, Gerald J. Friedman and Sol Sherry.

From the Department of Medicine, New York University, and Bellevue Hospital.

The vitamin C requirement was determined on 2 male adults who were hospitalized during the study and fed diets containing constant minimal amounts of vitamin C. The 24-hour urinary excretion of vitamin C was determined daily and the plasma concentration 3 times a week. The determination of vitamin C in both plasma and urine was done by the indophenol titration method[1] in the first case and in the second case both by titration and by the photoelectric colorimeter.[2, 3] The use of the photoelectric colorimeter reduces the error due to the small amount of non-vitamin C reducing substances present in the urine. On the diet fed, titration gave urine figures which were consistently higher by 10 mg per day. No significant differences were found in the plasma values.

The first case, L. R., age 57, height 72.5 in., weight 160 lb., was observed for a total of 110 days. In the first period of 19 days he received 50 mg of ascorbic acid daily; during the second period of 53 days he received 100 mg of ascorbic acid daily; during the third period of 22 days, 200 mg daily and during the fourth period of 16 days 350 mg daily. The ascorbic acid was given in divided doses of 50 mg each at regular intervals throughout the day. In the first period the daily excretion of vitamin C averaged 11 mg, the daily retention averaged 39 mg and the blood plasma concentration varied from 0.76 to 0.97 mg %, the average being 0.85 mg %. In the second period the daily excretion averaged 20 mg, the daily retention 80 mg, and the plasma vitamin C varied from 0.93 mg % to 1.22 mg %, the average being 1.12 mg %. In the third and fourth periods the vitamin C intake was increased to 200 mg and 350 mg respectively. The excretion increased but the amount retained did not go above an average of 91 mg daily. The plasma concentrations remained at the higher levels. Apparently the patient was retaining a maximal amount on an intake of 100 mg daily.

The second patient was 42 years of age, 66.5 in., weight 160 lbs.

* This investigation was aided by a grant from the Josiah Macy, Jr., Foundation.

1 Birch, T. W., Harris, L. J., and Ray, S. N., *Biochem. J.*, 1933, **27**, 590.

2 Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

3 Evelyn, K. A., Malloy, H. J., and Rosen, C., *J. Biol. Chem.*, 1938, **126**, 645.

The vitamin C studies were begun when the plasma concentration was 0.24 mg %. There was an interval of 2 months between periods II and III, during which time no observations were made. The patient was observed for a total of 179 days. In the first period of 6 days the patient was kept on diet alone, receiving no ascorbic acid; in the second period the patient received 100 mg of ascorbic acid daily for the 52 days. In the 3rd period of 8 days the patient was again kept on the diet alone; in the 4th period of 41 days he was given 50 mg of ascorbic acid daily; in period 5, 43 days, he received 75 mg of ascorbic acid; in period 6, 29 days, he was given 100 mg daily. The average daily excretion in period I was 6.4 mg; the plasma concentration was 0.24 mg %. In period II the average daily excretion was 6.0 mg daily; the retention was 94 mg daily; the plasma concentration rose to 1.06 mg %. A level of 1.00 mg % was reached in the plasma after 36 days. In the 3rd period of 8 days the plasma concentration fell from 0.56 to 0.27 mg %. In the 4th period of 41 days the daily excretion averaged 9.6 mg, the daily retention averaged 40 mg and the plasma concentration rose to 0.34 mg %, the average being 0.28 mg %. In the 5th period of 43 days the excretion averaged 7.9 mg, the retention averaged 68 mg daily and the plasma level rose to 0.75 mg %; the average for the last 20 days was 0.70 mg %. In the 6th period of 41 days the daily excretion averaged 10 mg; the daily retention was 90 mg. The plasma level rose to 1.43 mg % and was maintained at an average of 1.23 mg % for the last 17 days.

From these observations it seems that the optimum intake of vitamin C daily is 100 mg. At this intake the blood plasma concentration will be maintained at or above a level of 1 mg %. If a greater amount of vitamin C is fed it is excreted in the urine.

TABLE I.
Average Daily Excretion of Vitamin C in 2 Adults.

Period No. of Days	Ascorbic acid fed daily, mg	Avg daily excretion Vitamin C, mg ± S.D.	Avg daily retention Vitamin C, mg	Plasma levels Vitamin C, mg %
		Case I.		
I-19	50	11 ± 4.6	39	0.85
II-53	100	20 ± 2	80	1.12
III-22	200	109 ± 20	91	1.14
IV-16	350	259 ± 48	91	1.15
		Case II.		
I- 6	none	6.4 ± 3.9		0.24
II-52	100	6. ± 4.4	40	0.20-1.06
III- 8	none	6.1 ± 2.0		0.56-0.27
IV-41	50	10. ± 5.2	40	0.28
V-43	75	7.9 ± 3.6	68	0.30-0.73
VI-41	100	10. ± 3.7	90	0.73-1.43

10509 P

Experimental Induction of a Disorder Resembling Toxemia of Pregnancy in the Rabbit.

H. S. N. Greene.

From the Rockefeller Institute for Medical Research, Princeton, N. J.

A clinical and pathological investigation of a spontaneous and highly fatal intoxication in pregnant rabbits indicated that the disorder was of endogenous origin and that it was analogous to toxemia of pregnancy in women.[1, 2] The occurrence of the disease in typical form in pseudopregnant and in post-partum animals eliminated the possibility of a toxic factor arising from the products of conception. On the other hand, the presence of productive changes in the pituitary in contrast to the degenerative character of lesions in other organs suggested that an abnormal secretory activity might be a primary factor in the genesis of the intoxication. Accordingly, attempts were made to induce the disorder in experimental animals with various pituitary preparations.

Pituitary extracts were prepared by first grinding the anterior lobes of fresh beef glands with sterile sand to form a paste. After the addition of 3 cc of distilled water per gram of anterior lobe tissue, the mixture was agitated for 6 hours in a mechanical shaker. Following centrifugation, the supernatant fluid was withdrawn and used immediately or stored for a brief interval at freezing temperature.

Subcutaneous or intraperitoneal injections of 5 cc of this extract, 500 rat units of Antuitrin S* or 1 cc of Antuitrin* made on the 27th day of gestation and repeated on the 29th day induced a disorder which resembled the spontaneous disease in many particulars. On the other hand, control inoculations of sterile saline or fresh and autolyzed extracts of beef muscle and liver failed to reproduce any feature of the condition.

The clinical pictures and pathological changes induced by beef pituitary preparations, Antuitrin S and Antuitrin were similar but, in general, a less severe disorder followed the administration of Antuitrin S than occurred after injections of the other materials. The severity of the induced disease was directly proportional to the incidence of spontaneous toxemia of pregnancy in the family group used and the different clinical types of the spontaneous disorder were

1 Greene, H. S. N., *J. Exp. Med.*, 1937, **65**, 809.

2 Greene, H. S. N., *J. Exp. Med.*, 1938, **67**, 369.

* Parke, Davis & Company.

duplicated in almost every respect in experimental animals of different genetic constitution. In some instances, loss of appetite and slight indisposition were the only manifestations, while in others the picture was that of a profound intoxication with excessive salivation, thirst, acetone breath and prostration the cardinal signs. The blood pressure was always decreased and a drop to 30 mm of mercury from a preinoculation level of 80 mm commonly followed the administration of the beef pituitary preparation.

The chemical blood changes were less pronounced but were in general agreement with those observed in the spontaneous disease. The most marked alterations from normal were the presence of acetone, elevation of non-protein nitrogen, urea nitrogen and creatinine, decrease of calcium and increase of inorganic phosphate. The uric acid value was not consistently changed.

The disorder following a single injection on the 27th day of gestation occasionally terminated in death, while that induced by 2 injections given on alternate days resulted in death in approximately 40% of cases. At autopsy, animals that died or were killed 2 to 3 days after the last injection showed changes comparable with those observed in spontaneous toxemia of pregnancy and in addition, the ovaries contained large, hemorrhagic follicles. The predominating changes were found in the liver, kidneys, and adrenal glands. Microscopically, the liver showed widespread, intense, fatty degeneration and areas of necrosis which were sometimes peripheral in distribution but were usually scattered without constant relation to the zones of the lobule. The kidney showed pronounced fatty changes and, occasionally, bilateral cortical necrosis was found but acute inflammatory changes were never noted. Fatty degeneration occurred in all portions of the adrenal and frequently a homogeneous, fatty, necrotic zone entirely separated the cortex and the medulla. As a rule, the adrenal changes were most pronounced and the liver and kidney lesions least marked in animals that died after a short, clinical disorder, while the reverse held in animals that died or were killed after a longer illness.

At the present time, aqueous anterior lobe extracts in various concentrations and dosages have been administered in 45 pregnant rabbits and a disorder comparable to one of the clinical types of spontaneous toxemia of pregnancy resulted in 95.5% of cases. In like manner, the administration of Antuitrin S to 13 pregnant animals gave positive results in 84.6% of cases. Antuitrin has been used in 4 experiments and toxemia resulted in all instances.

The spontaneous intoxication is not identical with the toxemias of

pregnancy in man but the rôle of the 2 diseases in pregnancy is the same and such differences as exist may well be of a generic order. In like manner, there are some differences between the spontaneous and experimental intoxications in the rabbit which may be attributable to the fact that in one case the disease is of gradual evolution while in the other it is precipitated abruptly. The evidence so far obtained is sufficient to warrant further investigations of the possible rôle of the pituitary in the etiology of the human disorder.

10510

Rate of Elimination of Divinyl Ether.

WILLIAM L. RUIGH.* (Introduced by I. S. Ravdin.)

From the Research Laboratories, Merck & Company, Rahway, N. J.

We have found that the rate of elimination of divinyl ether from an anesthetized dog does not follow the formula first developed for acetone by Widmark[1, 2] $C_t = C_o.e^{-V\lambda t/m}$ where C_o is the original concentration of volatile substance in the blood; C_t the concentration after time t; t, the time; V, the alveolar ventilation; λ, the partition coefficient between air and blood; and m, the "reduced volume". A mathematical treatment has also been applied to the elimination of ethyl ether by Henderson and Haggard.[3, 4] Widmark's simplified formula is $C_t = C_o.e^{-\alpha t}$ where α is the "elimination constant" and which in the logarithmic form becomes $\log C_t = \log C_o - \alpha t$. If, therefore, the rate of elimination of a substance which follows this formula is plotted on semi-logarithmic paper with $\log C_t$ plotted against t a straight line should be obtained the slope of which is determined by the elimination constant.

Curve I in Fig. 1 is the typical straight line obtained for ethyl ether with dogs based on the data of Haggard.[4] Curve II, also from Haggard's data, shows the effect of administering carbon dioxide on the rate of elimination by increasing the alveolar ventilation. At point (A) on the curve the administration of carbon dioxide was stopped and the subsequent portion is essentially parallel to Curve I.

* Present address, Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, Pa.

1 Widmark, E. P., *Acta Med. Scandinavica*, 1919, **52**, 57.

2 Winterstein, H., *Die Narcose*, Berlin, 1926, p. 220.

3 Henderson, Y., and Haggard, H. W., *Noxious Gases*, New York, 1927

4 Haggard, H. W., *J. Biol. Chem.*, 1924, **59**, 753.

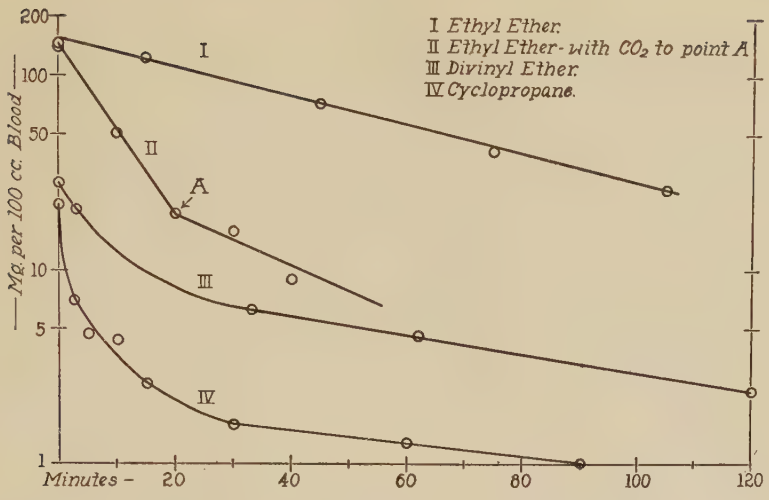


FIG. 1.

Elimination of anesthetics from the blood of anesthetized dogs. Concentration of anesthetic in the blood is plotted on a logarithmic scale against the time in minutes.

Curve III shows a result obtained in our laboratory for divinyl ether (the technic of these blood concentration studies will be published elsewhere). This curve does not become a straight line until after 30 minutes and hence we must infer that some new factor is in operation which is not accounted for by the Widmark expression. Comparing divinyl ether with ethyl ether, it will be seen that the initial rate of elimination of divinyl ether is much greater corresponding to the earlier recovery of consciousness from divinyl ether anesthesia. This could be predicted from the Widmark formula since for divinyl ether λ^1 is 0.76[5] and for ethyl ether 0.065.[4]† Later, however, the rate of elimination of divinyl ether is slower and finally is even less than that of ethyl ether.

The elimination of cyclopropane is shown by Curve IV, which is based on the recent data of Robbins.[6] The remarks made for divinyl ether also apply here. When the results of Nicloux[7] on chloroform are plotted in this way a straight line is obtained over a period of 6½ hours subsequent to the first 30 minutes period. The fact that

5 Ruigh, Wm. L., and Erickson, A. E., in press.

† Widmark's λ is the reciprocal of λ the Ostwald solubility expression which is numerically equal to the partition coefficient water-air. The differences in values obtained with blood are disregarded.

6 Robbins, B. H., *Anesthesia and Analgesia*, 1937, **16**, 93.

7 Cited in Meyer-Gottlieb, *Experimental Pharmakologie*, 8th edition, Berlin, 1933, p. 123.

the elimination of chloroform follows an exponential formula after 30 minutes was first noted by Widmark.[1]

The explanation of this behavior, we believe, can be reached by a consideration of the influence of the lipoid-blood or as actually measured, the olive oil-water partition coefficients of the anesthetics. The partition coefficients of acetone, 0.23,[8] and ether, 3.2,[9] are of a different order of magnitude from those of cyclopropane, 34.2,[9] divinyl ether, 41.3,[5] and chloroform, 100.[10] The overall rate of elimination of the first class, represented by ethyl ether, and the *initial* rate of the second class of lipotropic anesthetics is undoubtedly governed by the factors of alveolar ventilation, pulmonary blood flow and the air-water distribution ratio as stressed by Widmark and Haggard. We believe that the final rate of elimination, after an initial 30 minutes' transition period of the second class of anesthetics, is determined primarily by the slow rate of diffusion of the substance from the masses of depot fat in the body. Such fat has a poor blood supply and acts as a reservoir for the anesthetic. The elimination again follows a logarithmic curve but the causative factors differ from those mentioned earlier.

This concept of the mechanism of elimination is in entire harmony with the observation of Seevers, Meek, Rovenstine and Stiles,[11] that large quantities of cyclopropane are slowly "lost" in closed spirometer experiments with dogs. Their preferred explanation lay in the high lipoid solubility of this gas although they mention the possibility of destruction by metabolism and slow diffusion through the skin.

In conclusion it may be pointed out that with an anesthetic having a high oil-water partition coefficient, the final rate of elimination is governed by 2 practical factors—the duration of the anesthesia and the relative amount of fat present in the body. With the lipotropic anesthetics, chloroform, divinyl ether or cyclopropane, the prolonged administration to obese persons should be undertaken with caution if the long-continued presence of residual anesthetic in the patient be considered undesirable.

---

[8] *International Critical Tables*, III, p. 425.

[9] Orcutt, F. S., and Seevers, M. H., *J. Pharm. and Exp. Therap.*, 1937, **59**, 206.

[10] Lindenburg, A., *Compt. rend. soc. biol.*, 1933, **112**, 1524.

[11] Seevers, M. H., Meek, W. J., Rovenstine, E. A., and Stiles, J. A., *J. Pharm. and Exp. Therap.*, 1934, **51**, 1.

10511

Further Studies on Therapeutic Properties of Sulfapyridine* in Experimental Pneumococcus Infections.†

L. H. Schmidt and Carolyn Hilles. (Introduced by S. Tashiro.)

From the Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

An earlier report[1] showed that sulfapyridine (2-sulfanilamidopyridine) had a curative action when administered to mice infected with Type XXII pneumococci. This pneumococcus was one of 11 types which had been shown[2] to be refractory to sulfanilamide therapy. The greater effectiveness of sulfapyridine noted above, and a similar finding by Whitby[3] in Type I infections, suggested that this drug might have curative properties in infections with other types of pneumococci which were refractory to sulfanilamide therapy.

The present report concerns the therapeutic properties of sulfapyridine in experimental infections with pneumococci Types I, II, III, IV, V, VI A and B, VII, VIII, XI, XX, XXIV, XXVII and XXIX. These pneumococci include 10 of the 11 types which were refractory to sulfanilamide therapy and in addition Types I, V and VII which responded favorably.[2]

Groups of white mice were infected intraperitoneally with 100 to 1000 lethal doses of a given type pneumococcus.‡ Some of these mice served as untreated controls. The remainder received sulfapyridine 2 hours after infection and at repeated intervals thereafter according to one of the following schedules: *A*—20 mg sulfapyridine 2, 8, 14 and 20 hours after infection and every 24 hours thereafter for 5 days—least intensive therapy; *B*—20 mg of sulfapyridine

* Supplied through the courtesy of Dr. David A. Bryce, The Calco Chemical Company, Bound Brook, N. J.

† Supported in part by a grant from the Union Central Life Insurance Company, Cincinnati, Ohio.

1 Hilles, C., and Schmidt, L. H., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 73.

2 Schmidt, L. H., and Hilles, C., in press.

3 Whitby, L. E. H., *Lancet*, 1938, **1**, 1210.

‡ For stock cultures of these pneumococci we are indebted to Miss Annabel Walter, Bureau of Laboratories, Department of Health, New York City, and to Dr. Mary Kirkbride, New York State Department of Health, Albany. All of the pneumococci used in this work were of highest virulence; 1 to 8 pneumococci constituted a lethal dose and this number of organisms was invariably contained in 10^{-8} or 10^{-9} cc of a blood broth culture incubated for 12 hours at 37°C. The technique of increasing the virulence and determining the lethal dose has been described elsewhere.[2]

every 6 hours for 4 days and then every 12 hours for 2 additional days—treatment used if *A* failed to cure 50% of the infected mice; *C*—20 mg of sulfapyridine every 6 hours for 7 days and then every 12 hours for 3 additional days—most intensive therapy, used if *B* failed to cure 50% of the infected mice. In all experiments the drug was administered orally as a 10% suspension in 10% acacia. At the conclusion of treatment cultures of tail blood were made. Mice were considered cured if they survived 30 days after infection and had negative heart blood cultures at the end of that time.

Table I summarizes the results in infections with the 13 types of pneumococci mentioned above and with Type XXII.[1] The experiments shown are those in which the best therapeutic response has been obtained. Other experiments with all of these 14 types of pneumococci, except I and VII, have shown that poorer results are obtained when mice receive less intensive treatment than that shown in the table; mice that received sulfapyridine always lived longer than untreated controls, but few of the treated mice recovered. For comparison Table I includes a summary of the results of sulfanilamide therapy in infections with the same strains of pneumococci (data from earlier experiments[2]).

Table I shows that sulfapyridine was most effective in Types I and VII infections; the least intensive of the various treatments (A) led to the recovery of all the infected mice. The drug was next most effective in infections with Types IV, V, VI A and B, XI, XXII, and XXIX, but in order to effect cures in 50% or more of the mice, intensive therapy (B) had to be maintained for 4 days. Types XX, XXIV, and XXVII infections were more refractory to treatment than any of the above, but 30% of the mice infected with Type XX and 50 and 85% of those infected with Types XXIV and XXVII, respectively, recovered when intensive sulfapyridine therapy (C) was continued for 7 days.

Sulfapyridine was least effective in infections with pneumococci Types II, III, and VIII.§ Although the most intensive therapy (C) prolonged life, it did not lead to the recovery of a significant number of animals. As judged by its life-prolonging action, the drug had a greater effect in Type II infections than in either Types III or VIII. Fourteen of the 20 mice, infected with Type II pneumococci and treated with sulfapyridine, survived the 10-day period of therapy;

§ It is worth noting that the preliminary clinical study of Flippin and his coworkers[4] indicated that Type III infections responded least well to sulfapyridine therapy.

[4] Flippin, H. F., Lockwood, J. S., Pepper, D. S., and Schwartz, L., *J. A. M. A.*, 1939, **112**, 529.

TABLE I.

Therapeutic Effectiveness of Sulfapyridine in Experimental Pneumococcus Infections.

Type of pneumococcus	Infecting Dose: No. of lethal doses	Infecting Dose: cc of 12-hr culture	No. of mice in group	Sulfapyridine treatment	No. of Deaths Days After Infection: 1-5	6-10	11-15	16-30	Survivors: No.	%	Results of Sulfanilamide Therapy† Avg surival time, Days
I	100	10^{-7}	24	A*	0	0	0	0	24	100	(67 % of mice recovered)
	,,	,,	20	0	20	0	0	0	0	0	
II	100	10^{-6}	20	C*	1	5	13	0	1	5	9.4
	,,	,,	20	0	20	0	0	0	0	0	
III	100	10^{-6}	20	C*	1	17	0	0	2	10	4.4
	,,	,,	20	0	20	0	0	0	0	0	
IV	1000	10^{-6}	20	B*	1	7	0	0	12	60	9.8
	,,	,,	10	0	10	0	0	0	0	0	
V	100	10^{-6}	20	B*	0	4	3	0	13	65	(67 % of mice recovered)
	,,	,,	10	0	10	0	0	0	0	0	
VIA	100	10^{-6}	20	B*	1	4	0	0	15	75	11.7
	,,	,,	20	0	20	0	0	0	0	0	
VIB	100	10^{-7}	20	B*	0	2	0	0	18	90	12.2
	,,	,,	20	0	20	0	0	0	0	0	
VII	1000	10^{-6}	20	A*	0	0	0	0	20	100	(92 % of mice recovered)
	,,	,,	10	0	10	0	0	0	0	0	
VIII	100	10^{-6}	20	C*	0	16	1	0	3	15	7.0
	,,	,,	20	0	20	0	0	0	0	0	
XI	100	10^{-7}	20	B*	3	2	0	0	15	75	12.2
	,,	,,	20	0	20	0	0	0	0	0	
XX	1000	10^{-6}	20	C*	0	4	10	0	6	30	9.2
	,,	,,	20	0	20	0	0	0	0	0	
XXII	100	10^{-6}	20	B*	4	2	0	0	14	70	7.6
	,,	,,	20	0	20	0	0	0	0	0	
XXIV	1000	10^{-6}	20	C*	1	3	6	0	10	50	11.4
	,,	,,	20	0	20	0	0	0	0	0	
XXVII	100	10^{-7}	20	C*	0	2	1	0	17	85	7.3
	,,	,,	20	0	20	0	0	0	0	0	
XXIX	1000	10^{-6}	20	B*	1	4	0	0	15	75	7.8
	,,	,,	10	0	10	0	0	0	0	0	

*A. 20 mg sulfapyridine 2, 8, 14 and 20 hours after infection and every 24 hours thereafter for 5 days.
B. 20 mg sulfapyridine 2 hours after infection and every 6 hours thereafter for 4 days and then every 12 hours for 2 days.
C. 20 mg sulfapyridine 2 hours after infection and every 6 hours thereafter for 7 days and then every 12 hours for 3 days.

†Results with most effective treatment: 10 mg sulfanilamide, subcutaneously, 2 hours after infection and every 6 hours thereafter for 8 days and then every 12 hours for 2 days.

these animals were apparently in good health at the conclusion of treatment; 13 of them had positive blood cultures, however, and died between the 11th and 16th days after infection.

Our observations in Type VIII infections do not support Whitby's conclusion[3] that sulfapyridine is especially effective in infections with this pneumococcus. The discrepancy might have resulted from the use of different strains of organisms; more probably it was due to Whitby having terminated his experiments (those with Type I excepted) 7 days after infection. Our experiments show that this period of observation is too short to warrant a conclusion of curative action unless supported by data on blood cultures; at least 80% of our mice infected with pneumococci Types II, III, and VIII survived 7 days, but very few have recovered.

Table I also shows that sulfapyridine is a more effective therapeutic agent than sulfanilamide in infections with all of the 14 types of pneumococci investigated. A similar observation had been made by others[3, 5] in Types I and II infections. It is interesting to note that infections with Types I and VII responded most readily to both sulfapyridine and sulfanilamide, and infections with Types III and VIII were the most difficult to treat. In view of the similarity in the relative effectiveness of the 2 drugs, one may predict that infections with the remaining 16 types of pneumococci will respond favorably to sulfapyridine therapy—since these types responded to sulfanilamide as did Types I and VII.[2]

The fact that sulfapyridine therapy was not equally effective in infections with all types of pneumococci seems especially important in so far as the clinical use of the drug is concerned. It suggests that infections with certain types of pneumococci may require much more intensive treatment than infections with other types, and that infections with some types may be refractory to sulfapyridine. If these suggestions are correct, it will be particularly important to evaluate the clinical usefulness of the drug on the basis of results obtained with each of the 30 types of pneumococci.

Summary. Experiments have shown that sulfapyridine has a curative action when administered to mice infected with pneumococci Types I, IV, V, VI A and B, VII, XI, XX, XXII, XXIV, XXVII, and XXIX. The drug has little curative action in infections with Types II, III, and VIII, although it does prolong life.

[5] Cooper, F. B., Gross, P., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 87.

10512

Sulfanilamide and Sulfapyridine in Treatment of Experimental *B. typhosus* (*Eberthella typhosus*) Infections of Mice.

John A. Kolmer and Anna M. Rule.

From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Buttle, Parish, McLeod and Stephenson[1] found sulfanilamide in dose of 0.025 g by oral administration somewhat effective in the treatment of mice inoculated intraperitoneally with 100 lethal doses of *B. typhosus* (3 strains) when given immediately after inoculation in a single dose, or when the drug was given in the same dose twice daily; of 20 treated mice 15 were alive 12 days later while all of 10 untreated controls died within 2 days. When mucin was used as a diluent for the culture, protection was obtained against a slightly larger number of fatal doses of culture. Ten mice infected with 500,000 organisms survived for 12 days when treated with 2 daily oral doses of 0.025 g sulfanilamide. In one experiment in which treated mice were observed for one month, no deaths occurred after the twelfth day. A single oral dose of 0.025 g shortly after infection was found almost as effective as the same oral dose repeated twice daily for 6 days, but when treatment was delayed 5 hours after the infection, the result was not as good as with immediate treatment, although some protection was observed. Taking 25 g as the average weight of the mice employed, the dose of 0.025 g per mouse was equivalent to approximately 1.0 g per kilo. Schmidt[2] has reported improvement in 3 cases of typhoid fever following treatment with prontosil 1. One of these, thought to be dying, improved in 24 hours and recovered in 6 weeks.

Our experiments were conducted with a motile strain of *B. typhosus (Eberthella typhosus)* isolated about 8 months previously from a case of typhoid fever. The minimal lethal dose of 24-hour broth culture by intraperitoneal inoculation was approximately 0.1 cc (Table I) and 0.2 cc by intraperitoneal inoculation was employed in treatment experiments with sulfanilamide and sulfapyridine.

Since difficulty was experienced in giving mice accurate doses by mouth, the compounds were administered in solution by subcutaneous or intraperitoneal injection. For this purpose 0.4 g of sulfanilamide was dissolved in 50 cc of hot saline giving 0.008 g per cc; 0.2 g

1 Buttle, G. A. H., Parish, H. J., McLeod, M., and Stephenson, D., *Lancet*, 1937, **1**, 681.

2 Schmidt, J., *Munch. Med. Wchn.*, 1936, **83**, 2122.

TABLE I.
Virulence of *B. typhosus* for Mice.

Wt, g	Dose,* cc	Results in Days					
		1	2	3	4	5	6
26	0.005	—	—	—	—	—	—
25	0.005	—	—	—	—	—	—
28	0.01	—	—	—	—	—	—
24	0.01	—	—	—	—	—	—
21	0.05	—	—	—	—	—	—
23	0.05	—	—	—	—	—	—
19	0.1	—	—	D			
22	0.1	—	—	—	D		
23	0.1	—	—	D			
25	0.2	—	—	D			
22	0.2	—	D				
24	0.2	—	—	D			
26	0.3	D					
21	0.3	—	D				
20	0.4	D					
22	0.4	D					

* Of 24-hour broth culture by intraperitoneal inoculation.

sulfapyridine was dissolved in 200 cc of hot saline solution giving 0.001 g per cc.

In one experiment summarized in Table II, 6 mice were given 2 doses of 0.160 g *sulfanilamide* by *subcutaneous* injection 6 hours apart before intraperitoneal inoculation with 0.2 cc of 24-hour broth culture followed by a third dose 12 hours thereafter and repeated at 6-hour intervals. Two additional mice were kept as untreated infection controls and both succumbed in 48 to 72 hours after inoculation. Two more were included as drug controls and survived 12 days when

TABLE II.
Effect of Sulfanilamide by Subcutaneous Injection in Treatment of *B. typhosus* Infections of Mice.

Wt, g	Dose per kilo,* g	Results in Days								
		1	2	3	4	5	6	8	10	12
24	.160	—	—	—	—	—	D			
25	”	—	D							
25	”	—	—	—	D					
23	”	—	—	D						
26	”	—	—	—	—	D				
24	”	—	D							
24	”	—†	—	—	—	—	—	—	—	—
26	”	—†	—	—	—	—	—	—	—	—
23	Control	—	D							
25	”	D								

*By subcutaneous injection; 2 doses given 6 hours apart before intraperitoneal inoculation with 0.2 cc of 24-hour broth culture of *B. typhosus* followed by third dose 12 hours thereafter and repeated at 6-hour intervals.

†Drug controls.

the experiment was terminated. Of the 6 treated animals, 4 survived for 1 to 2 or 3 days beyond the untreated controls, indicating that sulfanilamide in the dose administered probably showed some therapeutic effect although the results were not as encouraging as those reported by Buttle and his colleagues, probably because the dose employed was about 6 times smaller per kilo of weight.

In a second experiment 8 mice were inoculated intraperitoneally with 0.2 cc of 24-hour broth culture; 2 kept as untreated controls died in 48 to 72 hours. Six of the mice were given 0.160 g *sulfanilamide* by *subcutaneous* injection immediately after inoculation and the dose repeated every 6 hours; 1 survived for about 4 days but the remaining 5 succumbed in 2 to 3 days after inoculation (Table III); 2 drug controls lived for 12 days, when the experiment was terminated.

In a third experiment 14 mice were inoculated intraperitoneally with 0.2 cc of 24-hour broth culture; 4 kept as untreated controls died in 24 to 72 hours. Ten of the mice were given 0.160 g *sulfanilamide* by *intraperitoneal* injection 2 hours after inoculation with a second dose 4 hours later and thereafter at 6-hour intervals. Of these 3 survived 12 days, while the remaining 7 succumbed within 4 days (Table IV); 2 drug controls survived 12 days, when the experiment was terminated.

In a fourth experiment 14 mice were inoculated intraperitoneally with 0.2 cc of 24-hour broth culture; 4 kept as untreated controls died in 24 to 48 hours. Ten of the mice were given 0.169 g *sulfapyridine* by *intraperitoneal* injection immediately after inoculation with a second dose 4 hours later and thereafter at 6-hour intervals. Of

TABLE III.

Effect of Sulfanilamide by Subcutaneous Injection in Treatment of *B. typhosus* Infections of Mice.

Wt, g	Dose per kilo,* g	Results in Days								
		1	2	3	4	5	6	8	10	12
22	.160	—	—	D						
25	”	—	D							
25	”	—	D							
24	”	—	—	—	D					
22	”	—	D							
26	”	—	D							
24	”	—†	—	—	—	—	—	—	—	—
24	”	—†	—	—	—	—	—	—	—	—
22	Control	D								
24	”	—	D							

*By subcutaneous injection begun immediately after intraperitoneal inoculation with 0.2 cc of 24-hour broth culture of *B. typhosus* and repeated every 6 hours.

†Drug controls.

these one survived for 12 days, while the remaining 9 succumbed in 24 to 72 hours (Table V); 2 drug controls survived 12 days, when the experiment was terminated.

TABLE IV.

Effect of Sulfanilamide by Intraperitoneal Injection in Treatment of *B. Typhosus* Infections of Mice.

Wt, g	Dose per kilo,* g	Results in Days								
		1	2	3	4	5	6	8	10	12
22	.160	—	—	—	—	—	—	—	—	—
24	”	—	D							
25	”	—	D							
21	”	—	—	D						
24	”	D								
24	”	—	D							
25	”	—	—	—	—	—	—	—	—	—
26	”	—	—	D						
23	”	—	—	—	—	—	—	—	—	—
22	”	—	—	—	D					
25	”	—†	—	—	—	—	—	—	—	—
25	”	—†	—	—	—	—	—	—	—	—
25	Control	—	D							
24	”	—	—	D						
21	”	D								
22	”	D								

*By intraperitoneal injection. First dose 2 hours after inoculation with 0.2 cc of 24-hour broth culture of *B. typhosus;* second dose 4 hours later and repeated at 6-hour intervals.

†Drug controls.

TABLE V.

Effect of Sulfapyridine by Intraperitoneal Injection in Treatment of *B. Typhosus* Infections of Mice.

Wt, g	Dose per kilo,* g	Results in Days								
		1	2	3	4	5	6	8	10	12
23	.160	—	D							
22	”	D								
24	”	—	—	D						
24	”	—	—	—	—	—	—	—	—	—
24	”	—	D							
23	”	D								
25	”	D								
25	”	—	D							
22	”	—	—	D						
24	”	—	D							
20	”	—†	—	—	—	—	—	—	—	—
24	”	—†	—	—	—	—	—	—	—	—
22	Control	D								
20	”	—	D							
25	”	D								
22	”	D								

*By intraperitoneal injection. First dose immediately after inoculation with 0.2 cc of 24-hour broth culture of *B. typhosus;* second dose 4 hours later and repeated at 6-hour intervals.

†Drug controls.

Summary. Sulfanilamide by subcutaneous injection in dose of 0.160 g per kilo apparently prolonged the lives of some mice when given in 2 doses 6 hours apart before intraperitoneal inoculation with *B. typhosus* in a dose fatal in about 48 hours, followed by subsequent doses at 6-hour intervals. But when the compound in the same dose was given by subcutaneous injection immediately after inoculation followed by subsequent doses at 6-hour intervals there was much less evidence of therapeutic activity. However, sulfanilamide was much more effective when given intraperitoneally in the same dose 2 hours after intraperitoneal inoculation followed by a second dose 4 hours later and subsequent doses at 6-hour intervals. Of the total of 22 treated mice in 3 experiments, 3 survived and the lives of 7 were prolonged, whereas all of 8 untreated controls succumbed in 1 to 2 days after inoculation.

Sulfapyridine was less effective. Of 10 mice given 0.160 g by intraperitoneal injection immediately after inoculation, followed by a second dose 4 hours later and subsequent doses at 6-hour intervals, one survived and the lives of 2 were prolonged about 24 hours beyond the survival of 4 untreated controls which succumbed in 24 to 48 hours after inoculation.

10513

Sulfapyridine and Sulfanilamide in Experimental Pneumococcal, Meningococcal, Welch Bacillary and Friedländer's Bacillary Infections in Mice.*

ELEANOR A. BLISS, W. HARRY FEINSTONE, ALICE W. GARRETT AND PERRIN H. LONG. (With the technical assistance of Earl Ott.)

From the Biological Division, Department of Medicine, The Johns Hopkins University.

We[1, 2] have discussed the comparative therapeutic efficiency of sulfapyridine and sulfanilamide in the treatment of experimental hemolytic streptococcal and Type I pneumococcal infections in mice. These reports were based upon results obtained in mice the origins of

* This investigation was supported by a grant from the Chemical Foundation, Inc., of New York City.

1 Long, P. H., Bliss, E. A., and Feinstone, W. H., *Penna. Med. J.*, 1939, **42**, 483.

2 Long, P. H., *J. A. M. A.*, 1939, **112**, 538.

TABLE I.

Comparative Therapeutic Effects of Sulfapyridine and Sulfanilamide in Control of Experimental Pneumococcal, Meningococcal, Welch Bacillary and Friedländer's Bacillary Infections in Mice.

Organism	No. Mice	Inoculum M.L.D.	Drug	Deaths—Days After Infection											Survivals	
				1	2	3	4	5	6	7	8	9	10	10-15	No.	%
Pneu. I	25	630	S.P.	10	9	4	1								1	4
,,	25	,,	S.	24	1											
,,	15	,,	Control	15												
Pneu. II	50	890	S.P.		7	13	10	5	7	6	1	1				
,,	50	,,	S.	6	41	2	1									
,,	30	,,	Control	30												
Pneu. III	50	1040	S.P.	1	4	6	8	2	1	25	2	1				
,,	50	,,	S.	2	5	5	1	17	11	9						
,,	30	,,	Control	30												
Meningococcus	50	10,000	S.P.	30	11	1									8	16
,,	50	,,	S.	14	22	1			1						12	24
,,	30	,,	Control	30												
Welch Bacillus	40	—	S.P.	23	1										16	40
,,	40	—	S.	24	3										13	32
,,	40	—	Control	36	1										3	7
Friedländer's Type B	45	1603	S.P.	1			1		1	20	9	2	8	3		
,,	45	,,	S.			9	9	17	1	4						
,,	30	,,	Control	24	6											

S. = Sulfanilamide.
S.P. = Sulfapyridine.
Treatment—Pneumococcal and Friedländer's infections, 10 mg *per os* T.I.D. for 5 days, B.I.D. for 1 day, Q.D. for 1 day. Meningococcal and Welch bacillary infections, 10 mg just after infection.

which were unknown. Recently, we have used pure bred mice (strain CF1) whose genetic formula is ccaabb, as test animals for the study of the effects of chemotherapeutic agents upon various types of experimental infections. We believe the use of pure bred mice eliminates the factor of variations in host susceptibility.

In this report we will present data concerning the comparative therapeutic effects of sulfapyridine and sulfanilamide upon experimental infections in mice produced by several types of microörganisms. With the exception of the Welch bacillus and meningococcus, the strains of organisms were virulent for mice in dilutions of 10^8 to 10^9, and the meningococcus was made highly virulent by suspension in mucin. The technic of producing these infections has already been described by us.[3, 4] The data presented in each instance represent the average of several experiments.

As will be noted in Table I, the chemotherapeutic effect of sulfapyridine in experimental Types I, II, and III pneumococcal infections in mice is superior to that of sulfanilamide. The results obtained by us are distinctly inferior to those reported by Whitby,[5] but this may be explained by the higher mouse virulence of the strains of pneumococci which we employed and the fact that we used slightly smaller, though more frequently repeated, doses of sulfapyridine.

Little difference was noted in the chemotherapeutic effects of the two compounds in the control of experimental meningococcal or Welch bacillary infections in mice. In experimental Friedländer's bacillary infections in mice sulfapyridine maintained about the same margin of superiority as a therapeutic agent over sulfanilamide as was noted in the instance of the pneumococcal infections.

In conclusion, we may say that while sulfapyridine is superior to sulfanilamide in its therapeutic efficiency in the control of experimental pneumococcal and Friedländer's bacillary infections in mice, its effect does not approach that noted when either drug is used in the treatment of experimental hemolytic streptococcal infections in mice. Hence, it should be borne in mind that while sulfapyridine gives definite indications of being a useful drug in the treatment of pneumococcal pneumonia in human beings, it does not represent the ultimate goal in the chemotherapy of pneumococcal infections.

---

[3] Long, P. H., and Bliss, E. A., *Canad. Med. Assn. J.*, 1937, **37**, 457.

[4] Feinstone, W. H., Bliss, E. A., Ott, E., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 565.

[5] Whitby, L. E. H., *Lancet*, 1938, **1**, 1210.

10514

A Flocculative Reaction of *Perfringens* Toxin and Antitoxin.

A. J. Weil and C. H. Parsons. (Introduced by M. Heidelberger.)
From the Lederle Laboratories, Inc., Pearl River, N. Y.

The assay of *Perfringens* antitoxin depends entirely on tests on the living animal. The only *in vitro* method used is the antihemolysin test, which, however, deals with a fraction of the antitoxic activity often considered of minor importance (Prigge,[1, 2] Le Fort, and Molina;[7] compare, however, the dissenting opinions of Dalling[3] and Weinberg.[4] We are studying this question of parallelism between general antitoxic power and antihemolytic qualities at the present time). Besides the hemolysin there is no toxic function of *Cl. welchii* (type A) known which would give a sure indication of a multiplicity of toxins.

The object of this communication is to report a flocculation of *Perfringens* toxin and antitoxin which seems to parallel the flocculation of other toxins with their antitoxins, such as that of diphtheric toxin-antitoxin.

The main feature of our method is the use of concentrated toxin.* The unconcentrated toxin shows no visible flocculation or even a turbidity if mixed in any usual proportion with the antitoxin. Filtrates of 18-hour broth cultures having a potency of about 50 MLD per cc (intravenous test on mice) were concentrated 16- to 35-fold by ultrafiltration with 8% Parlodion membranes. Slight turbidity of some of the concentrates was removed either by filtration or centrifugation.

The antitoxins used were either unrefined or refined sera of horses being hyperimmunized against *Perfringens* toxin. The refined sera were prepared by Parfentjev's method of partial peptic digestion.[5] The majority of the sera tested contained antitoxin against other

1 Prigge, R., *Z. Immunitätsf.*, 1936, **89**, 477.

2 Prigge, R., *Ibid.*, 1937, **91**, 456.

3 Dalling, T., and Ross, H. E., *J. Comp. Path. and Ther.*, 1938, **51**, 235.

4 Weinberg, M., Nativelle, R., and Prévot, A., *Les Microbes Anaérobes*, Paris, 1937, Masson Cié.

5 Parfentjev, I. A., 1936, U.S. Patent 2065, 196; further data, *J. Immunol.*, 1938, **35**, 399.

7 Le Fort, M. P., and Molina, G., *Rev. Inst. Bact. de Chile*, 1937, **6**, 49.

* The use of concentrated toxin to obtain a flocculative reaction was previously applied by Rane and Wyman in their work on streptococcal toxin.[8]

8 Rane and Wyman, *J. Immunol.*, 1937, **32**, 321.

toxins of the gas-gangrene group and some also tetanal antitoxin, as indicated in Table II.

After some preliminary tests the following procedure was adopted: Sera were diluted with saline solution and used in decreasing amounts, the intervals between being about 20% and the volume being made up to 1 cc. The degree of dilution depends of course on the antibody-

TABLE I.

Example of Toxin-antitoxin Flocculation.

1 cc of *Perfringens* toxin (concentrated 30-fold) was mixed with decreasing amounts of diluted serum as indicated below. Readings were made after 60 to 75 m. incubation at 45°C.

Test tube	Serum cc	Saline cc	Antitoxin 14, diluted 1/5	Antitoxin, 18, diluted 1/5	Antitoxin 28, diluted 1/20	Antitoxin 23, diluted 1/20
1	1.0	—	t	t	t	—
2	0.8	0.2	tt	tt	t	t
3	0.65	0.35	tt	tt	+	tt
4	0.5	0.5	+	(+)	++	++
5	0.4	0.6	t	++	+++	++
6	0.3	0.7	(t)	+++	+	t
7	0.25	0.75	—	++	t	(t)
8	0.2	0.8	—	t	(t)	—
9	0.15	0.85	—	—	—	—
10	0.12	0.88	—	—	—	—
11	0.1	0.9	—	—	—	—
12	—	1.0	—	—	—	—

Data on antitoxins used are given in Table II.
(t), t, tt: degree of turbidity.
(+), +, ++, +++: degree of flocculation.

TABLE II.

Comparison of Mouse Units and Flocculative Units of *Perfringens* Antitoxins.

Antitoxin Unrefined	Mouse-units	Flocculative-Units	Antitoxin Globulin Modified	Mouse-units	Flocculative-units
1. Univalent	50	55	19. Bivalent	700	700
2. "	50	55	20. "	1300	1125
3. "	200	240	21. Multivalent	700	840
4. Bivalent*	50	50	22. "	750	840
5. "	50	50	23. "	750	800
6. "	170	180	24. "	800	830
7. "	105	100	25. "	800	750
8. "	200	180	26. "	800	830
9. "	300	275	27. "	850	750
10. "	300	275	28. "	850	900
11. "	300	360	29. "	900	1000
12. "	600	600	30. "	900	900
13. "	270	250	31. "	950	1000
14. Multivalent†	180	175	32. "	1000	830
15. "	225	230	33. "	1000	950
16. "	275	260	34. "	1000	840
17. "	275	225	35. "	1100	1100
18. "	300	300			

*Bivalent: contains antitoxins against *Perfringens* and *Vibrion septique*.
†Multivalent: contains antitoxins against *Cl. welchii, Vibrion septique, Cl. novyi, Cl. sordelli, Cl. histolyticum*.

content of the serum. In our series of tests, sera below 100 mouse-units[6] were diluted ½, sera between 100 and 500, 1/3 or 1/5, and sera above 500 diluted 1/10 or 1/20. For unknown sera a preliminary test with 100% intervals should be used to find the proper range. Tests with 100% intervals are also helpful in finding the proper range for a new lot of toxin. The addition of 1 cc of concentrated toxin was found suitable in 3 out of 4 toxins. One toxin, concentrated only 16-fold, was used in 2 cc amounts. The mixtures were thoroughly shaken and incubated at 45°C in a waterbath. Turbidity appears rather quickly, particulation becoming visible after 1 to 4 hours. Flocculation-time varies more from serum to serum than between different lots of toxin. Readings were made as soon as flocculation appeared. Where 2 tubes showed the same degree of flocculation the value was calculated by interpolation. The flocculative unitage, X, was computed by the equation $X = S_2U/S_1$, where S_1 = amount (cc) of serum in "indicating" tube, S_2 = amount of standard serum tested in "indicating" tube, U = flocculative units of standard serum allotted arbitrarily as equal to its unitage in mouse-protective units according to the official standard of the National Institute of Health.[6] In 131 tests a very satisfactory correlation between results of the flocculation and mouse-test[6] was obtained. Table II gives the results with the toxin most frequently used. Numerous controls (normal sera, antitoxins against *Vibrion septique,* antibacterial sera, and antitoxic sera other than those of the bacteria causing gas gangrene) were added. None of them showed any trace of reaction.

The flocculate is transparent, gelatinous, similar to the antidiphtheric, but less copious. It sinks quickly, forming a loose agglomerate which is readily redispersed by shaking. This type of particulation is very characteristic. With 3 out of 4 lots of toxin there was only one zone of flocculation. The fourth also reacted in this way during the first month after being made. Later tests showed with some sera a coarse precipitation appearing in lower dilutions of the sera than those which gave the typical flocculation. The type of flocculation alone was sufficient to distinguish this from the correct zone; however, we think that toxin-concentrates showing this phenomenon should not be used in routine work. We are not able to give any explanation for this "coarse precipitate" at the present time.

The investigation of the practical usefulness of the test continues. Agreement on standardization should not be difficult.

The lack of purity of the concentrates with which we are working

[6] Bengtson, I. A., *Public Health Rep.*, 1934, **49**, 525.

at present makes it rather difficult to give any data on the theoretical side of the problem. The close agreement of protective and flocculative values indicates that the flocculation is a true toxin-antitoxin reaction. The agglutinative titer showed no correlation with the flocculative value.

The ratio of serum to toxin as shown by our tests indicates that the actual amount of toxin present in the unconcentrated filtrate of *Cl. welchii* is very small. This would mean that the low toxic qualities of such filtrates—low in comparison with other bacterial toxins—would not have to be ascribed to a relatively weak effect of the toxin but rather to a relatively small amount of toxin produced in broth cultures under conditions which we regard as optimal at the present state of our knowledge.

Summary. A flocculative reaction between the toxin of *Cl. welchii* (Type A) and its antitoxin is reported, which shows close correlation to the mouse-protective values of *Perfringens* antitoxins. Concentrated toxins are used as antigens.

10515 P

Calcium and Cephalin in Relation to the Clotting Power of Crystalline Trypsin.

JOHN H. FERGUSON AND B. NIMS ERICKSON.*

From the Departments of Pharmacology and of Pediatrics, University of Michigan, Ann Arbor.

It is true[1] that crystalline trypsin[2] can clot ordinary citrated plasma and can activate prothrombin without added calcium. As these experimental facts appear to conflict with the statement of Northrop and Kunitz[3] that a trace of ionized calcium is necessary, we have reinvestigated the point minutely, with an enzyme preparation kindly supplied by the Rockefeller workers.

It was found (Table I) that the trypsin is much more active in the presence of added calcium salt and that excess of citrate can inhibit its action. Whereas trypsin alone requires amounts of the order of 1-2 mg to coagulate 1 cc of citrated dog plasma and the clots quickly undergo fibrinolysis, much smaller quantities (0.01-

* Aided by a Sigma Xi research grant.

1 Eagle, H., *J. Gen. Physiol.*, 1937, **20**, 543.

2 Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 267.

3 Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1935, **18**, 456.

TABLE I.

	Trypsin 1:1000	Distilled water	Cephalin 1:1000	$CaCl_2$ N/10	Citrated plasma	Clotting-times (sec.) at 38°C	
	cc	cc	cc	cc	cc	A	B*
1.	.5	.5	—	—	1.0	150	270
2.	.5	.25	.25	—	1.0	110	225
3.	.5	.25	—	.25	1.0	40	50
4.	.5	—	.25	.25	1.0	20	20
5.	—	.75	—	.25	1.0	365	
6.	—	.5	.25	.25	1.0	80	

*Trypsin solution boiled for 5 min.

0.02 mg) of enzyme, *synergized by calcium,* produce better clots which do not lyse in 24-48 hours.

The addition of cephalin to clotting systems containing the proteolytic enzyme results in a minor improvement of the thrombic activity developed. For its full demonstration, this requires the presence of calcium.

A minor loss of activity on boiling the crystalline trypsin solution[4] is restored by added cephalin and calcium. It is known, of course, that cruder enzyme preparations are thermolabile.

Lung extracts evince a thromboplastic action exactly analogous to that of dilute trypsin (*plus* cephalin). The partial retention of the activity in boiled lung extract has been explained on the basis of its phospholipid content.[5] We have a semi-quantitative confirmation of this in tests made with the isolated (total) lung P-lipids. Pending isolation, may we not regard the major, thermolabile factor in thromboplastic tissue extracts as analogous to a weak proteolytic enzyme?

In contrast to the view[1] that trypsin activates prothrombin directly, the new data indicate that it acts via the accepted mode of thrombin formation,[6] with its recognized dependence on calcium. It is necessary to revise the view that diffusible calcium ions are essential, in favor of the hypothesis that ionization (or 'orientation') of calcium can occur at colloidal surfaces where the close juxtaposition of prothrombin, cephalin and calcium permits of the thrombin reaction[6] even in the presence of moderate amounts of citrate, heparin, etc.

A correlation between coagulation and clot-retraction and fibrinolysis gains support from an enzyme theory.

We suggest the term "thromboplastic enzyme" for all proteases which can be shown to aid blood clotting in a manner analogous to a *weak* trypsin solution.

[4] Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 367.

[5] Mills, C. A., *J. Biol. Chem.*, 1921, **46**, 135.

[6] Ferguson, J. H., *Am. J. Physiol.*, 1938, **123**, 341.

10516 P

Production of Antihormones by Prolonged Administration of Pituitary Extract. Effect on Anterior Hypophysis.

AURA E. SEVERINGHAUS AND KENNETH W. THOMPSON.

From the Department of Anatomy, Columbia University, and the Laboratories of Surgery and Physiological Chemistry, Yale University School of Medicine.

A cytological study has been made of the hypophyses of 2 dogs and 2 sheep which had received prolonged injections of extracts of sheep-pituitary glands,* and of 2 dogs that had been injected with antihormone-serum. The striking physical changes in one of the dogs, namely, an atrophy of the thyroid, adrenals and gonads, a cessation of growth, and the development of obesity, have been reported elsewhere.[1]

The 2 dogs which were injected subcutaneously with the sheep-pituitary extract for 4 and 7 months respectively, developed antihormones which inactivated not only the injected pituitary extract, but also several of the hormones of their own hypophyses.

The 2 dogs which were injected daily with 10 cc of canine antihormone-serum showed signs of moderate inactivation of the gonads, thyroids and adrenals.

Twin ewes were injected with sheep-pituitary extract† daily for 6 months. Their genitalia and mammary glands continually showed signs of activation, and at autopsy their gonads and uteri were found to be stimulated. No gonadotropic antihormone developed. A half-sister of these ewes served as a control.

The hyophyses of the dogs injected with sheep-pituitary extract. The basophile cells show the most striking abnormalities consisting of: (1) a great increase in the size and number of the basophiles; (2) an excessive clumping and liquefaction of cytoplasmic granules; (3) the formation of many vacuoles of 3 different types: (a) clear round spaces containing a non-stainable substance or one removed by the technic employed; (b) scattered clear pale blue vacuoles which in some of the basophiles coalesced to become extensive vacuolar

* Prepared by a slight modification of a method by van Dyke and Wallen-Lawrence.

1 Thompson, K. W., and Cushing, H., *Proc. Roy. Soc.*, B, 1934, **115**, 88.

† One ewe was injected with the van Dyke and Wallen-Lawrence extract, while the other was treated with an equal amount of an extract prepared from acetone-dried sheep hypophyses. This fact is important because of its relation to the matter of denaturation of the proteins in the extracts.

inclusions; (c) scattered deeply basophilic vacuoles which in some cells displaced the cytoplasm almost entirely.

In these widespread disturbances it is readily possible to demonstrate cells in which the vacuolation is identical to that of the typical castration cell, or again to that characteristic of total thyroidectomy. Still other cells show a combination of granular and liquefied areas which are indistinguishable from the hyalinization described by Crooke[2] as characteristic for the Cushing syndrome.

Certain acidophilic regions of the glands show small irregularly shrunken cells in varying stages of degranulation with pyknotic, deeply basophilic nuclei. Scattered among these are many chromophobic cells with exactly the same nuclear characteristics. These 2 cell types represent stages in an extensive reversion of acidophiles to chromophobes.

The hypophyses of the dogs injected with canine antihormone. These glands show 3 notable features: (1) a scarcity of the typical areas of chromophobe cells; (2) an almost total absence of normally granulated basophilic cells, many of the cells containing the hyaline material similar to that described by Crooke; and (3) marked hyperemia and edema.

In many of the basophile cells there is a large Golgi zone which together with an increase in mitochondria leads to the interpretation that these cells are active in the production and release of a secretory product. The predominance of these cells and the absence of chromophobes indicates an unusual force demanding maturity of basophiles. In other regions cells with the Crooke changes predominate.

There is without question an active degranulation of some basophilic cells into the edematous areas surrounding the capillaries or (in places) continuous with them.

The hypophyses of the injected ewes. The most striking change is a degranulation of the basophile cells in which mitochondria and Golgi apparatus are prominent. The acidophiles are increased in size, compactly granulated, and stain more brilliantly than do those of the control. Also there are areas with small acidophiles with pyknotic nuclei. These glands give cytological evidences in both the basophilic and acidophilic cells that the secretory activity is considerably increased over the normal.

The most important of the findings may be enumerated as follows: (1) The "Crooke changes" heretofore described only in the human pituitary gland have been experimentally produced in dogs, most prominently in the hypophyses of the dogs injected with the

[2] Crooke, A. C., *J. Path. and Bact.*, 1935, **41**, 339.

canine antihormone. (2) The changes in basophiles characteristic of both castration and thyroidectomy were observed in the hypophyses of the long-time injected dogs. (3) The presence of basophilic granules in the perivascular spaces and in the capillaries has been observed.

It is possible that the following reactions occurred in sequence during the course of prolonged injections of the sheep-pituitary extract: (1) An initial activation of the endocrine glands subsidiary to the pituitary gland occurred. In the sheep this process continued for the entire 6 months of injections. (2) The increased secretion of the subsidiary glands in turn activated the hypophyseal function. (3) The foreign protein linked to the injected sheep-extract gradually produced in the dogs a tissue and humoral resistance (antihormones), which ultimately inactivated the injected sheep-extract as well as certain pituitary hormones of the injected animal itself. (4) Thus, the antihormones produced a state of physiological hypophysectomy, which caused subsequent atrophy of the subsidiary glands. (5) The atrophy of the thyroid and gonads (and adrenals?) produced the final changes in the hypophyses characteristic of gonadectomy and thyroidectomy (and adrenalectomy?).

The Crooke changes characteristically found in the Cushing syndrome, associated with a diminished function of the gonads and thyroids, are believed to be related in some way to the effects of the inactivation of the subsidiary endocrine glands by the antihormone in these animals.

10517

A Case of Delayed Ovulation After Estrin Administration in the Intact Monkey.*

JOSEPHINE BALL AND CARL G. HARTMAN.

From the Phipps Psychiatric Clinic, Johns Hopkins Medical School, and the Department of Embryology, Carnegie Institution of Washington, Baltimore.

In the course of experiments designed to throw light on the hormonal basis of sex behavior a case of ovulation was encountered which was apparently delayed by the injection of estrin during the first part of the menstrual cycle. This occurred in monkey No. 634 which had been menstruating regularly (Sept. 22, Oct. 19, Nov. 19, Dec. 27, 1938, and Jan. 26, 1939). Ovulation, diagnosed by palpa-

* This work was supported in part by a grant from the Committee for Research in Problems of Sex, National Research Council.

tion, took place in each of these cycles; the exact date was not determined in the first cycle but in the last 3 cycles it occurred on days 11, 12 and 17, respectively.

Observations of the animal's sexual receptivity[1] had been started on November 23 and continued regularly 3 times a week until February 27, the date of laparotomy (Fig. 1). In spite of her apparently excellent physiological condition there had seemed to be none of the normal[1] tendency to increased sexual excitability in this animal at the time of ovulation. For this reason efforts were made to reproduce the normal picture by the administration of various hormones that have been found to produce or increase sex activity in castrates.

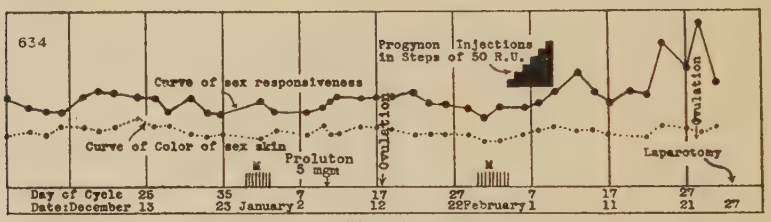


FIG. 1.

Progesterone (5 I.U. of Proluton Schering†) was given first, namely on January 5, day 10 of that cycle, without detectable effect on sex behavior, perhaps because the injection was made too long before ovulation took place.[2]

The following cycle estradiol benzoate (Progynon-B Schering) was tried in a dose that had produced in castrated female monkeys an increase in sexual responsiveness similar to that typically seen in normal animals at the time of ovulation.[3] This dose, as shown in Fig. 1, consisted of increasing daily injections for 6 successive days, starting on day 4 with 50 R.U. and augmenting the dose by 50 R.U. each day. This seemed to result in a slight increase in sex interest and in repression of ovulation. The left ovary had become slightly larger by day 14 but thereafter it remained stationary in size until day 27. As indicated in Fig. 1, the animal's sex interest was at this time rising slightly. As this was contrary to all previous experience with such tests at the end of the menstrual cycle, the ovarian changes were closely followed by palpation. Ovulation was diagnosed on day 29 (Feb. 23) and 4 days later observation at laparoto-

[1] Ball, J., and Hartman, C. G., *Am. J. Obst. and Gynecol.*, 1935, **29**, 117.

† We wish to thank the Schering Corporation for the Proluton and Progynon-B used in this experiment.

[2] Dempsey, E. W., Hertz, R., and Young, W. C., *Am. J. Physiol.*, 1936, **116**, 201.

[3] Ball, J., *Psychol. Bull.*, 1936, **33**, 811.

my confirmed this diagnosis. A fresh corpus luteum was found in the left ovary with a blood-red stigma 1 mm in diameter surrounded by an elliptical pink field 1.5 x 3 mm.

Of the 400 ovulations that have previously occurred in the Carnegie Rhesus colony, only 5 have taken place later than day 16. These 5 occurred on days 17, 19, 20, and 21 with a doubtful case on day 24. It seems, therefore, highly probable that the injection of estrin delayed ovulation in the present instance.

The rise of sex interest concomitant with the growth of the follicle and ovulation at the end of the cycle is not, however, to be attributed to the injections. This dose of estrin has been given repeatedly to some dozen castrates and the effect on sex behavior has always practically disappeared by the end of the week following the last injection. The increased sexual responsiveness probably occurred rather as a normal response to the animal's own hormones, this typical response having been inhibited earlier in the year by factors which, although unknown, seem, nevertheless, to have been operative in the whole group of 8 animals of which No. 634 was a member. Of the 6 other females in this group that were ovulating, 4 have shown improvement in sex responsiveness in February without reference to injected hormones; indeed, 2 of these have received no hormone treatment at any time throughout the year. It is evident that something was happening to the whole group which was improving their response to the mating situation.

Delay of ovulation does not regularly result from injection of estrogens during the early part of the cycle. Similar experiments have been made 3 times in another animal (No. 627), which was apparently in a similar physiological condition, without disturbing ovulation in the first and second cycles but completely inhibiting[4] it in the third cycle. In a third animal (No. 668), ovulation was inhibited (and sex behavior entirely uninfluenced) by the daily injection of 250 R.U. Progynon-B on days 8 to 12, increased to 500 R.U. on days 13 to 19 of the same cycle. However, the possibility of delayed ovulation suggested by the case here reported must be considered if estrin is to be used clinically to inhibit ovulation. It is also interesting to note this prompt recovery of the ovary from estrin inhibition.

The female under discussion was found to be menstruating on the morning of March 4, 9 days after ovulation.

Summary. Ovulation in a monkey was apparently delayed by administration of estrin in the first part of the menstrual cycle. Accompanying changes in sexual responsiveness are described.

[4] Corner, G. W., *Am. J. Physiol.*, 1935, **113**, 238.

10518 P

An Unidentified Growth Factor for Certain Strains of the Diphtheria Bacillus.

J. Howard Mueller.

From the Department of Bacteriology and Immunology, Harvard University Medical School.

It has been possible to grow a number of strains of the Park 8 variety of diphtheria bacillus, as well as one or 2 other unrelated strains, on media of entirely known composition.[1] Growth obtained on such media is extremely luxuriant, yet a number of the factors essential for the growth of organisms such as the staphylococci, the streptococci and the pneumococci are absent. It appeared, therefore, that such a medium might be unusually suitable for diagnostic purposes in preparing media for throat cultures to replace the ordinarily used Loeffler medium. A medium based upon Formula A in the paper referred to above has therefore been prepared, solidified by the addition of 2% agar, and used for the cultivation of organisms obtained by throat swab from a considerable number of individuals, both normal and suffering from clinical diphtheria.

The results in general, although encouraging, have been too unsatisfactory to suggest any immediate practical value for the method. The majority of normal throats yield no growth whatever after 24 hours' incubation. A certain number of cases of diphtheria, or cultures taken from the throats of individuals known to harbor the diphtheria bacilli, have given strongly positive growth of practically a pure culture of diphtheria. On the other hand, there are a certain number of normal throats which contain organisms capable of growth on this medium, a matter naturally to be expected, but there are also a number of cultures known to have been taken from positive throats which yielded no growth.

In following up this matter, it appeared that the difficulty lay in the fact that even with strains which grow well on the simple medium, a light inoculum generally fails to grow. This has been readily shown by streaking out cultures of suitable strains on agar plates prepared with such media as mentioned above. Growth is always heavy along the first streak of the inoculum, but later on—where one would expect single colonies to develop—no growth at all appears. In the course of 3 or 4 days' incubation, very frequently isolated colonies appear and grow to a good size. There is, how-

[1] Mueller, J. H., *J. Bact.*, 1938, **36**, 499.

ever, a definite and very long lag, and sometimes a complete failure to grow where the inoculum is light. A similar state of affairs appears to exist with the majority of freshly isolated strains which have been tested. They will grow with a comparatively heavy inoculum but not with a light one. Parallel streakings of the same material done in the same way on ordinary blood meat infusion agar give prompt growth, with single colonies developing over night. It is evident, therefore, that some essential factor for early growth, or for growth from small inocula, is absent from medium prepared according to the present formula.

This factor has been shown to be present in blood, where it would seem to be confined mostly to the serum. It will withstand a reasonable amount of autoclaving and of course may prove to be multiple. It has not been possible so far to show that it is identified either with Vitamin B 1 (thiamine), Vitamin B 2 (riboflavine), pantothenic acid, or mixtures of these substances. The nature of this factor is being investigated further, and the purpose of the present note is merely to indicate that the full story of the nutritional requirements of the diphtheria group of organisms is not yet completely cleared up.

10519

Absorption and Titration of Androgenic Hormone in Alcoholic and Oily Solutions Administered Percutaneously.

BERNHARD ZONDEK AND FELIX SULMAN.

From the Laboratory, Gynecological-Obstetrical Department, Rothschild-Hadassah Hospital, Jerusalem.

The percutaneous resorption of oestrone in oily solutions has been described.[1] Recently one of us[1] showed that the absorption through the skin of oestrogenic hormone in organic solvents is quantitatively fully equivalent to the subcutaneous absorption of oily solutions. As organic solvents, 96% alcohol, benzol, ether, benzene, acetone and many others may be used.

Ito, Hajazu and Kon[2] reporting similar experiments emphasized particularly the ready absorption of estrogenic hormone in 60% alcohol.

Our experiments with androgenic hormone were directed towards

1 Zondek, B., *Klin. Wschr.*, 1929, 2229; *Lancet*, 1938, 1107.

2 Masao Ito, Seizi Hajazu and Turuziro Kon, *Zbl. Gyn.*, 1937, **61**, 1094.

the detection of a method which, with the help of organic solvents, enabled us to determine amounts even smaller than was possible with the help of Fussgaenger's method.[3]

We attempted to determine minute amounts of androgenic hormone with the recovery test method using the technic described by Tschopp.[4] Male rats weighing 60-80 g were castrated and after 4 weeks subjected to treatment with androgenic hormone. For 10 days 3 groups, each consisting of 5 animals, received 27 gamma of androgenic hormone daily. In Group 1 the animals received the hormone dissolved in 0.1 cc of olive oil subcutaneously. In Group 2 the hormone was administered percutaneously dissolved in one drop of olive oil, and in Group 3 the percutaneous method was also used, but the solvent consisted of 1 drop of 96% alcohol. On the 11th day the animals were killed, the genital organs placed in 4% formalin (1:10) and weighed 24 hours later. Table I shows the average weights given in milligrams after treatment with a total of 270 gamma of testosterone.

Table I shows that the amount of 270 gamma of testosterone administered daily for a 10-day period is ineffective if given percutaneously in oily solution, that it causes an increase in weight in the male sexual organs of 220% if given subcutaneously in oily solution and a weight increase of 50% if given percutaneously in 96% alcohol. Similar results were achieved with testosterone acetate.

It is evident that the percutaneous administration of androgenic hormone in alcoholic solution is, indeed, superior to the percutaneous use of an oily solution, its effectiveness being, however, only about a quarter of that achieved by the subcutaneous administration of an oily solution.

In searching for an even more sensitive test animal than the rat, we decided to use the baby chick comb test described by Burrows, Byerly and Evans[5] and confirmed by Frank and Klempner[6] and by Frank, Klempner and Hollander.[7] This method was recommended by Danby[8] as well. We used 6-day-old chicks in groups of 10 respectively. The animals received over a 10-day period androgenic hormone in oily solution subcutaneously or intramuscularly, in other

[3] Fussgaenger, R., *Medizin und Chemie*, I. G. Farben, 1934, **2**, 194.

[4] Tschopp, E., *Arch. Internat. Pharmacodyn. et Thérap.*, 1936, **52**, 381.

[5] Burrows, W. H., Byerly, T. C., and Evans, E. I., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 50.

[6] Frank, R. T., and Klempner, E., *ibid.*, 1937, **36**, 763.

[7] Frank, R. T., Klempner, E., and Hollander, F., *ibid.*, 1938, **38**, 853.

[8] Danby, M., *Acta brev. Neerland*, 1938, **8**, 90.

TABLE I.

Weight of Genital Organs of ♂ Rats After 10 Days' Treatment with a Total of 270 Gamma Testosterone.

Solvent	Method of administration	Seminal Vesicle mg	Prostate mg	Cowper's gland mg	Preputial gland mg	Penis mg	Total mg	Proportion
Controls		15.3	34.7	38.7	35	71.3	195	1
Olive oil	percutaneously	14.3	31	35	49.7	69	199	1
" "	subcutaneously	76.6	145.7	182.7	79.3	149.7	634	3.2
Alcohol 96%	percutaneously	19.3	56.3	56	65	95.3	292	1.5

groups the combs were brushed with oily or alcoholic solutions. On the 11th day the animals were killed and the heads fixed for 24 hours in 4% formalin (1:10). Then the combs were dissected off and weighed. The average weight of the combs of the control animals (26 mg) was designed as 1, and the proportional enlargement was calculated from the average figures found in weighing the combs of the test animals. Table II shows the results of several series of experiments after treatment with testosterone.

TABLE II.
Weight of the Combs of Chicks After Treatment with Testosterone.

No.	Solvent	Method of administration	Dose of testosterone per day (gamma)	Total dosage of testosterone (gamma)	Wt of comb (avg mg)	Proportion (control = 1)
1	Controls	brushed (dry)	—	—	26	1
2	"	not treated	—	—	27	1
3	*Ol. arachidis*	subcutaneously	0.3	3	28	1
4	" "	intramuscularly	8	80	38	1.4
5	" "	"	16	160	57	2.1
6	" "	"	32	320	123	4.5
7	" "	percutaneously	0.15	1.5	27	1
8	" "	"	0.3	3	44	1.6
9	" "	"	0.6	6	52	2
10	96% alcohol	"	0.05	0.5	42	1.6
11	" "	"	0.1	1	47	1.8
12	" "	"	0.15	1.5	53	2
13	" "	"	0.3	3	73	2.7

Table II shows that by brushing the combs of chicks with alcoholic testosterone solution, determinaton of even 0.5 gamma (10 x 0.05, see example 10) is possible at least. Considering the quantities of hormone necessary to obtain a weight increase of the comb of 100%, if the percutaneous brushing of the comb is used, it is evident that about 4 times as much hormone in oily solution (No. 9) must be used, as that necessary in the alcoholic solution (No. 12) in order to obtain similar results. Considering the amount necessary to increase the weight of the comb by 60% we see that the oily hormone solution (No. 8) must contain about 6 times as much of the hormone as does the solution in 96% alcohol. (No. 10.)

The above experiments demonstrate that the test method which we propose for the detection of androgenic hormone is about 5 times as sensitive as that of Fussgaenger,[3] who brushes the combs of chicks with oily solutions. Based on the proportions indicated by Fussgaenger we can more or less establish the scale of sensitivity in comb units for testosterone shown in Table III.

The beforementioned experiments were carried out on a total of

TABLE III.

Solvent	Method of administration	Site of administration	Dosage (or multipla)
96% alcohol	Percutaneously	Comb	1
Oil	"	"	5
"	Subcutaneously	"	12.5
"	Intramuscularly	Glutaeus	250
"	Subcutaneously	Back	875

130 chicks, 10 chicks making up one series of assay. The average values obtained with reference to the size of the combs always represented clearcut results. In other series, however, now and then cases were observed in which a single chick showed an increase of the comb of more than 100%, compared with the average value of the others within the same series. On principle, we abstained in such cases from using such unhomogeneous experimental series. In any case we advise also to use for the baby chick comb test at least 10 chicks per group.[5–8]

With reference to the weight of the combs in the 17-day-old control chicks—at the termination of the experiment—we noted considerable variations of the average values depending on the season (12 mg in winter as compared with 30 mg in summer).

Summary. Studying the percutaneous absorption of androgenic hormone (testosterone) it was noted that the hormone dissolved in alcoholic solutions is more readily absorbed than that in oily solutions. Most instructive is this difference in the comb test, because here the hormone can be applied at the very site of its effectiveness.

Using the baby chick comb test, we found that brushing the combs with alcoholic solutions of testosterone is about 5 times as effective as brushing the combs with oily solutions, according to Fussgaenger's method. The method proved to be much more sensitive than the subcutaneous and intramuscular administration of the hormone since it is possible to detect quantities as small as 10×0.05 gamma $= 0.5$ gamma of testosterone at least. We are recommending the standardization of small quantities of androgenic hormone with the use of this method.

If the recovery test is used, on castrated rats, the effectiveness of the percutaneous method using alcoholic solutions of the hormone is less marked. As a matter of fact, alcoholic testosterone solutions are more readily absorbed than oily solutions percutaneously; in this method, however, the subcutaneous administration of the hormone in oily solution is the most effective (4 times as sensitive as the alcoholic percutaneous method).

10520

Adequacy of Cow Milk as a Source of Magnesium for Rats.

HARRY G. DAY AND ELSA ORENT-KEILES.

From the Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University.

Duncan, Huffman and Robinson[1] reported that calves restricted to whole milk diets alone, or supplemented with iron, copper and manganese, eventually manifest tetany which is indistinguishable from tetany in rats on a magnesium-low diet.[2] The tetany was prevented and serum magnesium was restored to normal by the administration of magnesium carbonate or magnesium oxide.[3] Since the content of magnesium in milk appears to be rather low, in relation to the calcium and phosphorus, it is possible that prolonged restriction to milk diets of young of other species might eventuate in magnesium deficiency, as suggested by Schmidt and Greenberg.[4] According to data cited by Cox and Mueller,[5] milk of cows and humans may contain about 0.013 and 0.005% magnesium respectively. Analyses of rat milk gave 0.031% magnesium.[5]

But in several investigations rats have been reared to adulthood on cow milk supplemented with iron and copper and in no instance have symptoms been reported which indicate the occurrence of magnesium deficiency.[6] This suggests that rats are either (a) better able than calves to utilize the magnesium of cow milk or (b) their requirement for the element is less than that of calves. Duncan and associates believed that " . . . . there is a failure in the magnesium metabolism which prevents the animal (calf) from utilizing the available magnesium." Whatever might be the explanation of these observations, the findings suggest that direct investigation should be made of the adequacy of cow milk as a source of magnesium for rats.

Young rats weighing 40 to 45 g were used. They were kept in individual cages and fed exclusively commercial grade A pasteurized cow milk *ad libitum*, supplemented with 0.25 mg thiamin per liter.

1 Duncan, C. W., Huffman, C. H., and Robinson, C. S., *J. Biol. Chem.*, 1935, **108**, 35.

2 Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1933, **100**, 603.

3 Huffman, C. H., and Duncan, C. W., *J. Dairy Sci.*, 1936, **19**, 440.

4 Schmidt, C. L. A., and Greenberg, D. M., *Physiol. Rev.*, 1935, **15**, 297.

5 Cox, W. M., Jr., and Mueller, A. J., *J. Nutr.*, 1937, **13**, 249.

6 Underhill, F. A., Orten, J. M., Mugrage, E. R., and Lewis, R. C., *J. Biol. Chem.*, 1932-33, **99**, 469.

Mineral supplements fed to all the rats were Mn, Fe and Cu at daily levels of 3.4, 2.4 and 0.2 mg respectively per rat. These were furnished as $MnSO_4 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$ and $CuSO_4 \cdot 5H_2O$. Twelve rats (A), sexes evenly divided, were given this basal ration supplemented with 0.194 g magnesium, as $MgSO_4 \cdot 7H_2O$, per 1000 cc of milk. This increased the content of magnesium approximately 100%, since, by analysis, the milk contained about 0.230 g magnesium per 1000 cc. Twelve other rats (B), similarly selected, were given the same basal ration and enough H_2SO_4 to equal the sulfate fed to rats A. After 56 days 4 of the rats in each group were sacrificed. Determinations were made of serum magnesium, bone ash and bone calcium and magnesium, using methods described by Orent, Kruse and McCollum.[7] On the seventy-fourth day the remaining rats were killed and the same determinations were repeated.

No symptoms were observed which suggested that the rats suffered any degree of magnesium deficiency. The average weights of males and females at the end of the study were 196 and 111 g respectively for those given the supplement of magnesium (Group A); whereas the weights of those without the supplement (Group B) were 207 and 112 g, respectively. The average weekly milk consumption of Group A was 375 cc and that of Group B was 370 cc. Marked variation was noted in the milk intake, a few rats ingesting 80 to 90 cc each per day while others took only 30 to 40 cc. The content of serum magnesium was not appreciably affected. The averaged value for Group A was 3.3 mg per 100 cc and that for Group B was 3.1. Similarly the bones showed no evidence of actual magnesium deficiency, although rats given milk fortified with magnesium had a larger percentage of bone magnesium than the unsupplemented controls (Table I).

In constructing Table I no distinction was made between values for rats killed after 56 days and those killed after 74 days, since the

TABLE I.

Calcium and Magnesium Content of Combined Femura, Tibiæ and Fibulæ of Rats on a Milk Diet, with (A) and without (B) Magnesium Supplementation.

	Bone ash per rat, g		% of ash			
			Ca		Mg	
	A	B	A	B	A	B
No. of analytical values	12	12	12	12	11	10
Mean	.8121	.8111	38.41	38.53	.729	.678
Standard deviation	.1688	.1766	0.50	0.33	.049	.039

[7] Orent, E. R., Kruse, H. D., and McCollum, E. V., *J. Biol. Chem.*, 1934, **106**, 573.

difference between the two was slight. The average percentage of bone magnesium in Group A rats was 0.714 after 56 days and 0.737 after 74 days. For Group B rats it was 0.676 after 56 days and 0.691 after 74 days, indicating that the content of bone magnesium was not decreasing as restriction to the milk diet continued.

Since, in addition to the normal serum magnesium and absence of deficiency symptoms, the magnesium content of bone did not continually decrease during the experimental period, it is concluded that cow milk contains enough magnesium to prevent magnesium deficiency in rats. This suggests that the magnesium requirement of rats is either (a) lower than that of calves, or (b) rats are better able than calves to utilize the magnesium of cow milk.

10521

Anticatalase Activity of Sulfanilamide and Related Compounds. III. Oxygen Tension and Bacteriostasis in Pneumococcal Cultures.

LAWRANCE E. SHINN, EDNA R. MAIN AND RALPH R. MELLON.
From the Western Pennsylvania Hospital Institute of Pathology, Pittsburgh, Pa.

In previous publications[2–5] the authors have investigated the theory, originally proposed by Locke,[1] that the retardation of growth of certain microörganisms in the presence of sulfanilamide may be primarily the result of the accumulation of hydrogen peroxide. This accumulation is presumed to arise through the inhibition of catalase by sulfanilamide which has been activated by oxidation. It was demonstrated[2] that sulfanilamide and many structurally related compounds have an appreciable anticatalase activity which is frequently enhanced by oxidative processes such as those involved in ultraviolet irradiation and[5] that bacteriostasis of Type I pneumococcus *in vitro* is accompanied by a correspondingly marked accumulation of hydrogen peroxide in the culture.

The formation of hydrogen peroxide requires time and oxygen,

1 Locke, A., Main, E. R., and Mellon, R. R., *Science*, 1938, **88**, 620.

2 Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 272.

3 Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1939, **36**, 183.

4 Mellon, R. R., *Modern Hospital*, 1938, **51**, 53.

5 Shinn, L. E., Main, E. R., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 591.

"points of sufficient importance as possible limiting factors in the application of sulfanilamide therapy to occluded and rapidly developing infection to warrant inquiry and test."[3] The dependence of the inhibitory power of sulfanilamide on oxygen has been examined experimentally *in vitro* with reference to the Type I pneumococcus and the results are herewith reported.

The cultures were grown in 50 cc Erlenmeyer flasks each containing 10 cc of veal-heart-infusion-broth, autoclaved immediately before use. Glucose (0.4%) and blood (5×10^{-5} cc) were then added to each flask. Sulfanilamide (10 mg %) was added where required. All flasks were seeded with 0.025-0.1 cc of a 6-hour broth culture of Type I pneumococcus, the exact amount for a given experiment being determined by the degree of growth of the seeding culture. Reduced oxygen tension was obtained by placing the flasks in vacuum jars and evacuating and refilling with nitrogen to atmospheric pressure until the required concentration of oxygen was theoretically present. In the last jar of each series an oxygen absorbent, sodium pyrogallate or hydrosulfite, was used to remove further traces of oxygen. All cultures were incubated 16-17 hours, at the end of which time growth was determined by turbidimetric comparisons. Plate-counts were avoided because of the chaining which occurs in the presence of sulfanilamide.

The results, given in Table I, show that decrease in oxygen tension is accompanied by decrease in the inhibition of growth caused by sulfanilamide. The average inhibition in the presence of air (21% oxygen) is 54%. At 10% oxygen the inhibition is 40% and the decrease continues until a concentration of 1% of oxygen is reached, where the inhibition is essentially zero. When the concentration is reduced to 0.04% there is uniformly a stimulation of growth in the presence of sulfanilamide. On still further reduction of the available oxygen by the presence of pyrogallate or hydrosulfite, the inhibition resulting from sulfanilamide reappears.

No hydrogen peroxide was detected (limit about 6 γ/cc) in the cultures which contained no sulfanilamide.* Peroxide was present in concentrations of 10-25 γ/cc in the sulfanilamide-containing cultures grown in 21 or 10% oxygen. No peroxide was detected in any cultures at the lower oxygen levels. This result accords well with the abrupt drop in inhibition (average) between the 10 and 5% oxygen levels.

It is difficult to explain the inhibition at low oxygen tension as a

* This would be anticipated from the constitution and relative old age of the cultures.

TABLE I.

Effect of Concentration of Oxygen in Gaseous Environment on Inhibition of Growth of Type I Pneumococcus by Sulfanilamide.

% Oxygen Present	Exp. 1			Exp. 2			Exp. 3			Exp. 4			Exp. 5			Avg I
	Gc	Gs	I	Gc	Gs	I	Gc	Gs	I	Gc	Gs	I	Gc	Gs	I	1-5
21	92	12	+87	102	31	+69	124	104	+16	72	21	+71	140	102	+27	+54
10	120	21	+82	142	102	+14	164	124	+24	140	86	+39	180	102	+43	+40
5	110	102	+ 7	142	102	+14	164	144	+12	102	102	0	120	102	+15	+10
2.5	102	102	0	112	112	0	164	144	+12	102	86	+16	140	120	+14	+ 8
1.0	102	102	0	122	92	+25	144	144	0	86	86	0	102	120	—18	+ 1
0.04	81	110	—25	62	92	—49	84	116	—36	76	86	—13	110	130	—18	—28
<0.04*	92	27	+71	72	32	+55	76	64	+16	62	13	+79	92	72	+22	+49

Gc—Growth in control culture.

Gs—Growth in culture containing 10 mg% of sulfanilamide.

$$\text{I—\% inhibition} = \frac{Gc - Gs}{Gc} \times 100.$$

*Oxygen tension reduced to 0.04% and an oxygen absorbent added to remove the remainder.

The growth unit employed is an arbitrary value based on turbidity-readings.

result of peroxide accumulation. As the oxygen tension reaches a sufficiently low level another mechanism may appear. While the anticatalase activity of sulfanilamide apparently depends upon activation of the *p*-amino group,[2] the sulfonamido group may become reactive under anaërobic conditions. Reduction to sulfides, known to be highly toxic, may account for the reappearance of inhibition under extreme anaërobic conditions (*cf.*, last line of Table I). The great volume of clinical and experimental work carried out with the sulfonamide compounds has shown that the presence of the sulfur group is essential to their effectiveness. Increased growth in the presence of sulfanilamide in the next to lowest oxygen concentration could be explained as stimulation by very small quantities of substances which are toxic in larger amounts. This condition of extreme anaërobiosis would probably not obtain clinically in pneumococcal or streptococcal infections.

Little attention has been given to the bacteriostasis of anaërobes or infections with anaërobes insofar as sulfanilamide is concerned. However, Spray[6] has shown that certain spore-forming anaërobes are susceptible to bacteriostasis by this compound. It may be that in such cases the anaërobic type of inhibition which has been demonstrated here will prove pertinent.

A significant comparison can be made between the concentration of oxygen required for adequate bacteriostasis and the concentration available *in vivo*. The oxygen present in the plasma of normal arterial blood is stated to be 0.6%.[7] Assuming the solubility of oxygen in broth to be essentially the same as in water and assuming that at the higher levels of oxygen concentration equilibrium is attained, the oxygen present in the cultures with atmospheres containing 21, 10, and 5% oxygen would be respectively 0.5, 0.24, and 0.12%. Thus under normal conditions the amount of oxygen present in plasma is adequate for support of a high degree of inhibition. The dependence of sulfanilamide upon oxygen indicates the advisability of maintaining or increasing, where possible, the oxygen supply at the lesion.

While this material was being prepared for publication there appeared 2 excellent articles which constitute essential confirmation, with respect to the streptococcus, of the mechanism which we have proposed. Fox, German, and Janeway[8] clearly demonstrated by

[6] Spray, R. S., *J. Lab. and Clin. Med.*, 1938, **23**, 609.

[7] Sollmann, T., *Manual of Pharmacology*, W. B. Saunders Co., Philadelphia, 1936, page 753.

[8] Fox, C. L., German, B., and Janeway, C. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 184.

potentiometric methods that cultures of the hemolytic streptococcus undergoing inhibition by sulfanilamide maintain an elevated potential indicating the presence of oxidizing substances. The normal growth in cultures without sulfanilamide was accompanied by a fall in potential. A similar fall in potential occurred in sulfanilamide-containing cultures after inhibition was overcome. The presence of cysteine or reduced access to oxygen diminished or obliterated the inhibitory power of sulfanilamide. The addition of catalase decreased the potentials attained and the degree of stasis. This effect of added catalase implies that the substance responsible for elevated potential and bacteriostasis is hydrogen peroxide. Fox and his coworkers draw no conclusion regarding the nature of the oxidizing substance. In view of the method employed, which is an extremely delicate but non-specific test for oxidizing and reducing agents, their caution is fully justified.

Warren, Street, and Stokinger[9] presented at the same time essentially similar results by the same methods. They have extended the study by testing some related compounds and showing that, among others, the presence of *p*-acetyl sulfanilamide, considered by most workers to be a completely inactive compound, caused no rise in potential and no inhibition.† Sulfapyridine produced inhibition and caused an elevated potential. In work soon to be published we have been able to show that *p*-acetyl sulfanilamide produces no inhibition and no peroxide accumulation in the pneumococcus and that sulfapyridine produces inhibition and peroxide accumulation in excess of that produced by sulfanilamide.

Warren, *et al.*, place a somewhat different interpretation on their results than do Fox and his coworkers. The divergence appears to be based largely on differing results with regard to the potential produced by suufanilamide in sterile broth. Variable findings on this point are not surprising in view of the fact that sulfanilamide could, in theory, cause such a rise under proper conditions. Traces of intermediate oxidation products of the *p*-amino group would probably confer an elevated potential. That these may exist in sulfanilamide solutions is indicated by the appreciable anticatalase activity of the non-irradiated compound.[2]

Summary. 1. Reduction of the percentage of oxygen in the

[9] Warren, J., Street, J. A., and Stokinger, H., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 208.

† In [2] it was shown that *p*-acetyl sulfanilamide is distinguished by possessing no anticatalase activity before or after irradiation.

superambient air of broth cultures of the Type I pneumococcus greatly reduced or prevented bacteriostasis by sulfanilamide.

2. When the oxygen concentration was reduced to 0.04% an actual stimulus of growth by sulfanilamide was found.

3. When the oxygen was further reduced by the presence of pyrogallate or hydrosulfite the inhibition by sulfanilamide reappeared.

4. Hydrogen peroxide was detected only in cultures in equilibrium with atmospheres containing 10% oxygen or more. These concentrations correspond to those permitting effective bacteriostasis and are comparable to those obtainable in the plasma.

5. The lack of inhibition at intermediate concentrations shows that oxygen plays a vital rôle in the action of sulfanilamide. The failure to form peroxide at these same concentrations is taken as evidence that oxygen exerts its influence through the agency of hydrogen peroxide. The stimulus and recurring inhibition at the lowest values is interpreted as evidence of the formation of a toxic reduction compound, possibly a sulfide. The latter type of inhibition may play a rôle in any bacteriostatic effect against anaërobes.

10522

Advances in the Serological Typing of *Streptococcus hemolyticus.*

ALVIN F. COBURN AND SUSAN O'CONNELL. (Introduced by M. H. Dawson.)

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

Griffith's[1] technic for typing Group A hemolytic streptococcus has been applied in this laboratory to the classification of more than one thousand strains recovered from a variety of infections. It was possible to type 70% of the organisms examined in 1935 and 1936 by the procedure described previously.[2] At that time several difficulties were pointed out: (1) the elimination of anti-C cross-reactions; (2) the granular character of matt organisms; and (3) the failure of certain strains to be agglutinated by any of the 28 available type-specific sera.

As shown previously[2] interfering cross-reactions due to anti-C substance in the sera could be eliminated by absorbing the sera with

1 Griffith, F., *J. Hyg.*, 1935, **34**, 542.

2 Pauli, R. H., and Coburn, A. F., *J. Exp. Med.*, 1937, **65**, 595.

purified C-substance, but the purification of C-substance according to Heidelberger's method was a matter of considerable difficulty. However, C-substance prepared by another method (Fuller[3]) has has proven quite satisfactory for this purpose[4] so that the preparation of type-specific sera has been greatly simplified.

The granular nature of the suspensions produced by many matt organisms interfered seriously with the typing of these organisms up to 1937. Subsequently two methods have been found to obviate this difficulty. Kodama[5] has made granular suspensions homogeneous by subjecting them to supersonic vibrations. In collaboration with Dr. L. Emmet Holt, Jr., we have confirmed this observation, but found the method impractical. A simpler alternative consists in subculturing granular organisms in broth containing trypsin. The trypsin (Difco-Bacto Trypsin supplied in solution in 10 cc ampoules) is stored at 5°C until used. One cc of the solution is added to 9 cc of phosphate-buffered broth *just prior to seeding*. A heavy inoculum of culture (about 0.1 cc) added to the mixture and incubated at 37°C usually gives satisfactory growth in 6 to 18 hours. Suspensions are then made in the customary way. Out of 188 matt strains which we had been unable to type with available methods, only 28 were still too granular for typing after one subculture in broth to which trypsin was added. Only 8 of these remained refractory on subsequent subculturing in this mixture.

The third difficulty, failure of agglutination with any of the 28 type-specific sera has been largely overcome by Griffith's addition of 2 new sera (type 29 "Coggins"; type 30 "Quinn"). Most of the strains which we previously had to classify as "no type" have fallen into one of these 2 types.

These modifications have made it possible for us to assign types to 90% of the Group A cultures examined since our previous report, about 900 in all. There are still 3 minor difficulties: (1) the sera of types 6 and 18 are weak; (2) types 15 and 17 are frequently difficult to distinguish and (3) a few strains are still not agglutinated by any of our 30 sera. Most of these "no type" strains were recovered during the early fall months from throat cultures of healthy subjects. It may be significant that the time of their isolation corresponds with the season of minimal activity of hemolytic streptococcus in New York City.

Type 30 was the predominant type of hemolytic streptococcus

3 Fuller, A. T., *Brit. J. Exp. Med. and Path.*, 1938, **19**, 130.

4 Little, Paul A., personal communication.

5 Kodama, T., *Kitasato Arch. Exp. Med.*, 1937, **16**, 245.

recovered by us in 1937 and 1938, accounting for 35% of our typed organisms. Types 13, 15, 25, 27 and 28, together accounted for 25%. Types 4 and 22, which had been predominant in 1935-36, were recovered only occasionally. Type 10 (N.Y.5) was the only type not recovered in New York City during these 4 years. In contrast, types 10, 8, and 1 were most prevalent in the throat flora of Tokyo during this period of study.[6]

10523 P

Concentration of Prothrombin in Blood of Babies (3 to 7 Days Old).

ARMAND J. QUICK AND ARTHUR M. GROSSMAN.

From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee.

The ease with which nutritional deficiency states develop in young infants suggests the advisability of studying their prothrombin level, since it is now known that vitamin K is required for the synthesis of this clotting factor.

Blood was obtained by venipuncture from normal healthy infants varying in age from 3 to 7 days. The prothrombin was quantitatively determined by the senior author's method[1] on both the undiluted plasma and on plasma diluted with an equal volume of saline solution. Typical cases of this series are presented in Table I.

The results plainly show that the prothrombin concentration in the blood of the babies studied was essentially the same as that of normal adult blood. It was found, however, that the prothrombin diminished in infants' blood more rapidly than from that of adults, and curiously in a few bloods, such as Case 3, the decrease of prothrombin was strikingly rapid when the plasma was diluted.

These findings are completely at variance with those reported by Brinkhous, Smith and Warner.[2] In their series the prothrombin concentration of the bloods of the 3 babies who were less than 11 days old were 27, 36, and 44% of normal. Furthermore, even in infants 2 months old, they found that the prothrombin was less than

6 Kodama, T., personal communication.

1 Quick, A. J., *J. A. M. A.*, 1938, **110**, 1658.

2 Brinkhous, K. M., Smith, H. P., and Warner, E. D., *Am. J. Med. Sci.*, 1937, **193**, 475.

TABLE I.

Case	Age of infant, days	Clotting time in seconds (Quick's method) Undiluted plasma	Plasma diluted with equal vol. saline sol.
1	3	11	15
2	3	11½	15½
3	3	12	16*
4	4	12	15½
5	4	12	15
6	5	11	15
7	5	11	15
8	7	12	15½
Normal control†		11-11½	15

*Clotting time after standing 5 minutes was 25 seconds.
†Adult blood.

50% of the adult level. In view of the exceedingly low incidence of hemorrhage in very young infants, such low prothrombin values seem rather surprising. It must be remembered that the methods of both Smith and Quick are empirical and based on certain unproved assumptions. Smith and his coworkers take for granted that high dilution of plasma does not alter the prothrombin, but does inactivate the normal antithrombin. The latter has been shown to be either serum albumin or a substance closely associated with this fraction,[3] and it is conceivable that there may be a sufficient difference in the serum proteins of the newborn from that of adults to modify the antithrombic action. Further study is necessary before it is possible to account for the singular results obtained by Smith and his coworkers. In view of the fact that the author's method has proved itself a sensitive and delicate test for determining prothrombin reduction in sweet clover disease,[4] vitamin K deficiency in the chick,[4] in the rat,[5] in chloroform poisoning,[1] and in certain cases of obstructive jaundice,[1, 6] it seems reasonable to believe that it would not fail to disclose a low prothrombin level in babies if such actually existed.

[3] Quick, A. J., *Am. J. Physiol.*, 1938, **123**, 712.

[4] Quick, A. J., *Am. J. Physiol.*, 1936, **118**, 260.

[5] Greaves, J. D., *Am. J. Physiol.*, 1939, **125**, 429.

[6] Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sci.*, 1935, **190**, 501.

10524 P

Chemotherapy of Experimental Rabies of Rats.

PAUL GROSS, FRANK B. COOPER AND MARION LEWIS.

From the Western Pennsylvania Hospital, Institute of Pathology, Pittsburgh.

Of the various viral diseases that have been investigated chemotherapeutically *lymphogranuloma inguinale* alone has responded uniformly favorably to treatment with certain aromatic sulfur compounds; a high percentage of cures has been obtained in both the experimental[1–4] and the clinical disease.[5–7]

The response of certain of other viral diseases to chemotherapy has been negligible or slight. Regarding canine distemper, just as many unfavorable[8–10] as favorable[11–13] papers have been published. The reported therapeutic action of 2,4-diaminoazobenzene-4'-sulfonamide in choriomeningitis of mice,[14] was not confirmed.[15–17] Presumably massive infections with influenzal virus were unaffected,[14, 15] whereas favorable results were claimed when the infecting dose was known to be small.[18, 19]

Chemotherapeutic experiments with the viruses of poliomye-

---

1 Levaditi, C., *Compt. rend. soc. biol.*, 1938, **128**, 138.

2 Levaditi, C., *Ibid.*, 1938, **128**, 875.

3 Levaditi, C., *Ibid.*, 1938, **129**, 490.

4 MacCallum, F. O., and Findlay, G. M., *Lancet*, 1938, **2**, 136.

5 Hamilton, G. R., *Military Surgeon*, 1938, **83**, 431.

6 Shaffer, L. W., and Arnold, E., *Arch. Dermatol. and Syphilol.*, 1938, **38**, 705.

7 Shropshear, G., *Illinois M. J.*, 1938, **74**, 153.

8 Dickerson, V. C., and Whitney, L. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 263.

9 MacIntyre, A. B., and Montgomerie, R. F., *Brit. M. J.*, 1938, **1**, 875.

10 Plummer, P. J. G., Mitchell, C. A., and Walker, R. V. L., *Can. J. Comparative Med.*, 1938, **2**, 139.

11 Dochez, A. R., and Slanetz, C. A., *Science*, 1938, **87**, 142.

12 Larsen, C. E., *J. Am. Vet. Med. Assn.*, 1938, **46**, 197.

13 Marcus, P. M., and Necheles, H., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 385.

14 Rosenthal, S. M., Wooley, J. G., and Bauer, H., *Pub. Health Rep.*, 1937, **52**, 1211.

15 Levaditi, C., *Compt. rend. soc. biol.*, 1938, **127**, 958.

16 Ronse, M., *Ibid.*, 1938, **127**, 845.

17 Findlay, G. M., and MacCallum, F. O., *Brit. M. J.*, 1938, **1**, 875.

18 Climenko, D. R., Crossley, M. L., and Northey, E. M., *J. A. M. A.*, 1938, **110**, 2099.

19 Oakley, C. L., *Brit. Med. J.*, 1938, **1**, 895.

litis,[20-22] fibroma and myxoma,[21, 22] encephalitis,[10, 14, 15] yellow fever and Rift-valley fever,[17] as well as foot-and-mouth disease[15] have yielded negative results.

The purpose of this report is to record briefly the results obtained with sulfanilamide* and sodium sulfanilylsulfanilate† in rats infected with fixed rabic virus.

The brain of a rat that died 7 days after intracranial infection with fixed rabic virus was macerated with 10 cc of saline under aseptic conditions. The resulting suspension was injected intracranially in 0.1 cc amounts into 29 etherized rats by the technic reported previously.[23] Treatments consisting of 150 mg of drug suspended in 0.5 cc of 15% gum acacia were given orally immediately following infection and repeated once daily for a total of 8 days.

TABLE I.
Mortality-rate of Treated and Untreated Rats Infected with Fixed Rabic Virus.

	Daily Dosage, mg	No. of rats	No. of rats dying on days following infection					
			5	6	7	8	9	10
Control	None	9	—	—	8	—	—	1
Sulfanilamide	150	10	—	1	3	—	4	2
Sodium sulfanilylsulfanilate	150	10	1	—	1	—	—	8

Table I shows that the drugs in the doses used were incapable of saving the lives of the infected animals, although a slight prolongation of life was obtained. These results are not necessarily conclusive of the impotency of the 2 drugs because no attempt was made to titrate the infectious material and because the infecting dose employed was unnecessarily heavy and the route of injection was "unnatural." The error of drawing conclusions from chemotherapeutic experiments in which the infecting dose has been improperly adjusted is well illustrated in certain papers reporting negative results with sulfanilamide-therapy of pneumococcal infections.

Conclusions. Against a heavy infecting dose of fixed rabies virus administered intracranially to rats, the oral administration of sulfanilamide or of sodium sulfanilylsulfanilate resulted in a slight prolongation of life but failed to save the life of any animal.

20 Toomey, J. A., and Takacs, W. S., *Arch. Pediat.*, 1938, **55**, 307.

21 McKinley, E. B., Acree, E. G., and Meck, J. S., *Science*, 1938, **87**, 43.

22 Rhodes, A. J., and van Rooyen, C. E., *Brit. Med. J.*, 1938, **1**, 924.

* Synthesized and donated to us by the Monsanto Chemical Co., St. Louis, Mo.

† Synthesized and donated to us by the Calco Chemical Co., Bound Brook, N. J.

23 Cooper, F. B., Gross, Paul, and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 835.

10525

Effect of Adrenalectomy on Rate of Fat Absorption.

RICHARD H. BARNES, ARNE N. WICK, ELMER S. MILLER AND EATON M. MACKAY.

From the Departments of Physiology and Botany, University of Minnesota, Minneapolis, Minnesota, and The Scripps Metabolic Clinic, La Jolla, California.

It has been thought for some time that the rates of absorption of glucose and fat from the intestinal tract are decreased after extirpation of the adrenals.[1, 2] This effect has been interpreted by Verzar and his coworkers[3] as being due to the absence of the hormone of the adrenal cortex which causes a postulated decrease in phosphorylation of both glucose and fat by the intestinal mucosa. According to this hypothesis, phosphorylation is necessary for the normal absorption of these 2 foodstuffs. Deuel, *et al.*,[4] and more recently MacKay and Clark[5] have found that adrenalectomy has no influence on the rate of absorption of glucose. Because of these differences it seemed desirable to reinvestigate the effects of adrenalectomy on the absorption of fats. Miller, *et al.*,[6] in a study of fat* absorption by means of "tagged" fat, found no significant decrease in the amount of phosphorylation of fed fat in the intestinal mucosa of adrenalectomized rats. This was accompanied by no decrease in absorption rate. As the fat fed in this study was in the form of methyl esters of fatty acids and as the amounts absorbed were only determined at 2 absorption periods, the following study was made, first to see if there is any difference between the rate of absorption of methyl esters of the fatty acids of corn oil and the natural fat,

1 Judovits, N., and Verzar, F., *Biochem. Z.*, 1937, **292**, 182.

2 Verzar, F., and Laszt, L., *Biochem. Z.*, 1935, **276**, 11.

3 Verzar, F., and McDougall, E. J., *Absorption from the Intestine*, Longmans, Green and Co., 1936.

4 Deuel, H. J., Jr., Hallman, L. F., Murray, S., and Samuels, L. T., *J. Biol. Chem.*, 1937, **119**, 607.

5 MacKay, E. M., and Clark, W. G., unpublished data.

6 Miller, E. S., Barnes, R. H., Kass, J. P., and Burr, G. O., PROC. SOC. EXP. BIOL. AND MED., 1939.

* Custom has more or less limited the use of the term "fat" to the glycerol esters of fatty acids. However, the glycerol esters of fatty acids which do not occur in nature are often referred to as fats and we have used the term "fat" here in the same broad sense (esters of naturally occurring fatty acids) rather than in reference to a compound of nature.

and second, to determine the effect of adrenalectomy on the absorption of these 2 substances.

Eighteen adult male rats, weighing from 200 to 270 g were bilaterally adrenalectomized. These animals and a comparable group of unoperated controls were fed on our stock diet and given 1.0% sodium chloride *ad lib.* for 4 days. Although their food intake was lower than that of the controls the operated animals were in good condition and exhibited no signs of adrenal "intoxication" such as a subnormal body temperature, malaise and so on. Other adrenalectomized rats to whom water instead of salt solution was supplied for drinking had their food intake reduced much more and the clinical condition of these animals was such as to make them unsuitable for the administration of fat. Even the apparently normal salt-treated adrenalectomized rats were prostrated by the administration of corn oil. The methyl esters of the corn oil fatty acids had a less deleterious effect. In view of the toxicity of these fats for the adrenalectomized organism the results obtained in our experiments become even more striking.

Feeders were removed while the salt solution for drinking was still supplied, and after 24-hour fasting, each rat was given 0.5 cc of fat per square decimeter body surface by stomach tube. Body surface was calculated by the formula of Carman and Mitchell.[7] Half of the animals received corn oil (Mazola) and the other half the methyl esters of the fatty acids of corn oil. Both of these 2 groups were further subdivided into adrenalectomized and unoperated control groups.

At 2, 4, and 6 hours after giving the fat each animal was anesthetized with ether and the abdomen opened. A hemostat was clamped at the junction of the esophagus and stomach and another at the pyloric junction of the duodenum. A third was then clamped at the cecal end of the small intestine and the intestine cut on the ileum side. This free end of the intestine was then stripped free of mesentery for about 3 inches and placed in an open flask. A large bore, dull needle which was connected by rubber tubing to a 5-gallon bottle containing 1.0% sodium chloride was then inserted into the duodenum just below the pyloric clamp. Pressure was introduced into the system by means of a pressure bulb connected to the sodium chloride reservoir, and the intestinal contents washed into the flask with about 150 cc of salt solution. The stomach and large intestine were then stripped open and washed separately. In some

[7] Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **76**, 380.

studies it was found necessary to make sure that none of the fed fat was retained in the intestine. Under such conditions the above described washing was followed with approximately 50 cc of 95% alcohol and this followed by 50 cc more of the salt solution. All of this procedure was carried out while the animals were anesthetized but alive. It required approximately 3 minutes including anesthetization for each animal.

In order to test whether any fat was absorbed by the villi, or trapped in the intestine, a loop was made by tying off the intestines of 3, live anesthetized rats. The loops were washed with warm saline and 1.5 cc of the methyl esters of conjugated fatty acids of corn oil, developed as a "tagged" fat by Miller, *et al.*,[6] injected into each. After 5 minutes the loops were washed as described above, the intestines stripped lengthwise and the mucosa scraped off. No "tagged" fat was found to be present in the content or adsorbed on the intestinal mucosa after this treatment.

The figures in Table I show the rates of absorption of methyl esters of the fatty acids of corn oil and unaltered corn oil in adrenalectomized, and unoperated control rats at 2, 4, and 6 hours after feeding. The results calculated on the basis of the weight of fat absorbed per square centimeter body surface during the different periods show no significant differences between the rates of absorption of the methyl esters and unaltered corn oil. Further, there is no significant difference between the rates of absorption for these fats in adrenalectomized and unoperated control rats. If the results are calculated on the basis of the approximate amount of fat absorbed per hour, as is shown in the last column in Table I, there is again no difference between any of the groups and the rate of absorption is the same for each hour after feeding. It is interesting to note the differences in the stomach emptying times. In all cases the methyl esters left the stomachs more rapidly than the corn oil. Even with this difference in emptying time the rates of absorption remained the same. This is contrary to the absorption of glucose where MacKay, Bergman and Barnes[8] found that the rate of absorption was roughly proportional to the amount available for absorption from the intestine. It is unfortunate that the data in Table I does not cover enough absorption periods to afford accurate calculations of the amounts of fat available for absorption each hour after feeding.

It is difficult to understand the difference between this study and

8 MacKay, E. M., Bergman, H. C., and Barnes, R. H., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 323.

TABLE I.

Absorption of Corn Oil (Mazola) and Methyl Esters of Fatty Acids of Corn Oil in Adrenalectomized and Unoperated Control Rats.*

Abs. Time hr	Fat fed	Treatment	Body surface, cm^2	g fed	Fat left in stomach, g	Fat left in intestine, g	Fat abs., g	Fat abs./ cm^2 B.S., mg	Fat abs./ cm^2 B.S./hr, mg
2	Corn oil	control	459	2.11	1.18	.58	.35	.76	.38
2	" "	adrenalectomized							
2	Me. ester	control	426	1.84	.52	1.07	.25	.59	.29
2	" "	adrenalectomized	415	1.84	.34	1.11	.40	.96	.48
4	Corn oil	control	424	1.93	.70	.60	.62	1.46	.37
4	" "	adrenalectomized	413	1.93	.76	.70	.47	1.35	.34
4	Me. ester	control	408	1.75	.54	.56	.64	1.57	.39
4	" "	adrenalectomized	409	1.75	.18	1.02	.54	1.32	.33
6	Corn oil	control	413	1.93	.32	.57	1.05	2.54	.42
6	" "	adrenalectomized	399	1.84	.46	.42	.97	2.43	.40
6	Me. ester	control	422	1.84	.26	.65	.93	2.20	.37
6	" "	adrenalectomized	410	1.84	.12	.52	1.19	2.90	.48

*Each figure is an average of a group of 3 animals.

those published by others on the effects of adrenalectomy on the absorption of fat. One essential difference does exist and that is the type of fat used. In most of his work, Verzar has employed olive oil. In the experiments presented here and in the study by Miller, *et al.*,[6] corn oil has been used. Steenbock, Irwin and Weber[9] have reported a great variation in the absorption of different fats. They found that 65% of fed olive oil was absorbed in 4 hours. Palm oil and oleo stock which were the slowest were only absorbed to about 36%. The data in Table I show approximately 31% corn oil absorbed in 4 hours. Other studies in this laboratory have also shown that olive oil is much more prone to cause diarrhea than corn oil and furthermore, doses of corn oil up to 3 cc have been given to adrenalectomized rats without any harmful effects. Verzar, and Laszt[2] have found that adrenalectomized rats receiving olive oil usually die within 12 to 24 hours after feeding. It might be that some such differences in the type of fat used will explain the apparent discrepancies in the effects of adrenalectomy on fat absorption. It is also possible that the secondary effects of adrenal insufficiency, not present in this series because of the salt regimen, account for the slower absorption of fat in adrenalectomized animals noted by earlier workers.

Summary. The methyl esters of the fatty acids of corn oil are absorbed at the same rate as unaltered corn oil. Adrenalectomy has no significant effect on the absorption of either of these two fats. The rate of absorption of both methyl esters of corn oil fatty acids, and unaltered corn oil is the same for each hour up to 6 hours after feeding.

[9] Steenbock, H., Irwin, M. H., and Weber, J., *J. Nutrition*, 1936, **12**, 103.

10526 P

Observations on Impermeability of Guinea Pig Placenta to the Foreign Protein Ricin, a Phytotoxin.

EMMETT B. CARMICHAEL AND LOUIS C. POSEY.

From the Department of Physiological Chemistry, University of Alabama, University, Alabama.

The placenta has been reported as permeable to such substances as amino-acids,[1] creatinine and creatine,[2] peptone,[3] insulin,[4] bacterial antibodies,[5, 6] and allergens.[7] Ascoli[8] showed that the placenta was impermeable to albumins, but he suggested the possibility of a proteolytic ferment which might aid in the transfer of these substances.

To further the study of placental permeability ricin was injected into a fetus of each of 30 pregnant guinea pigs. Ricin was dissolved in physiologic salt solution and injected in amounts which varied from 6 to 350 lethal doses[9] per kilo of the total weight of the pregnant mother. Saline solutions were injected into a fetus of each of 9 pregnant guinea pigs and this group served as controls. All of the pregnant guinea pigs were near full term and injections were made into the fetuses after laparotomy. Nembutal-ether anesthesia was used.

The mothers of the control group survived and pregnancy continued for an average of 10.4 days (6 hours to 20 days). All of the fetuses were normal at birth and they did not show any ill effects from the injection of the saline.

The results of the ricin-injected group are shown in Table I. One fetus in each litter was born dead and showed signs of ricin poisoning or one stillborn fetus was delivered too early to show characteristics of the poison, while the litter mates were alive at birth. One of the mothers gave birth to one fetus one day after operation and 3 other fetuses on the third day. One of the latter fetuses received the ricin and the mother lived. Three mothers were killed with an

1 Slemons, J. M., *The Nutrition of the Fœtus*, Yale University Press, 1919.
2 Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1918, **34**, 5.
3 Wertheimer, E., and Delezenne, G., *Compt. Rend. Soc. d. Biol.*, 1895, **10**, 191.
4 Carlson, A. J., and Drennan, F. M., *Am. J. Physiol.*, 1911, **28**, 391.
5 Staubli, C., *Centralbl. f. Bact. u. Parasitenk., Bd.*, 1903, **33**, 458.
6 Bourquin, Helen, *Am. J. Physiol.*, 1922, **59**, 122.
7 Rosenau, M. J., and Anderson, J. F., *J. Am. Med. Assn.*, 1906, **47**, 1007.
8 Ascoli, A., *Z. f. Phys. Chem.*, 1902, **36**, 498.
9 Carmichael, E. B., *J. Pharm. and Exp. Therap.*, 1929, **35**, 193.

TABLE I.
Results Obtained by Injecting Several Lethal Doses of Ricin into One Fetus of Each of 33 Pregnant Guinea Pigs.

No. of pregnant animals used	Hr after injection until delivery	Mothers	
		Lived	Died
15	4.5- 16	14	1*
7	30 - 40	3	2*
			2†
5	50 - 96	4	1†
6	108 -150	5	1†

*Wound sepsis—gave anesthetic 7-125 hours after delivery.
†Ricin and autogenous toxins following premature delivery. Death 10-60 hours after delivery.

anesthetic following wound infection and self-evisceration. Four mothers died 10 to 60 hours after deliveries. Three of these mothers showed hemorrhages characteristic of ricin poisoning, due, we believe, to the injections being made into the uterus. The remaining mother that died spontaneously retained her litter 5 days and lived about 30 hours after the delivery. The mother did not show the hemorrhagic signs of ricin poisoning and we believe that autogenous toxins from the uterus were responsible for her death.

One of the numerous theories of the etiology of eclampsia is the absorption of fetal proteins or isoagglutinins with the consequent production of anaphylaxis or an hemagglutinative reaction. McQuarrie[10] concluded that absorption of isoagglutinins did not occur in the intact placenta. Young[11, 12] suggested that toxic products were absorbed from infarcts of the placenta caused by premature placental separation. Boucek[13] confirms the conclusions of McQuarrie.

Our experiments with ricin support Ascoli, McQuarrie, and Boucek in demonstrating that the intact quinea pig placenta is impermeable to the toxic protein.

Conclusions. Although certain protein-like substances have been found to traverse the placenta, it seems that our experiments demonstrate that the guinea pig placenta is not permeable to the toxic protein ricin.

10 McQuarrie, I., *Johns Hopkins Hosp. Bull.*, 1923, **34**, 51.
11 Young, J., *Proc. Roy. Soc. Med., Lond.*, 1914, **7**, 307.
12 Young, J., and Miller, M. A., *Proc. Roy. Soc. Med., Lond.*, 1921, **14**, 247.
13 Boucek, C. M., *Am. J. Anat.*, 1928, **41**, 1.

10527 P

Production of Oligospermia in a Man by the Use of Testosterone Propionate.*

Norris J. Heckel. (Introduced by W. O. Thompson.)

From the Department of Urology, Rush Medical College, University of Chicago, and Central Free Dispensary.

This preliminary report deals with the effect on spermatogenesis of 10 to 25 mg of testosterone injected subcutaneously for 266 days in a 67-year-old man who was under daily observation for 17 months beginning August 9, 1937. The accompanying graph (Fig. 1) illustrates the effect on sperm counts.

The number of spermatozoa in the semen decreased during the administration of testosterone, increased after testosterone withdrawal, decreased when testosterone was again administered and increased after termination of the therapy.

It is of interest that recent experimental evidence appears to indicate that the male hormone may maintain, but not initiate spermato-

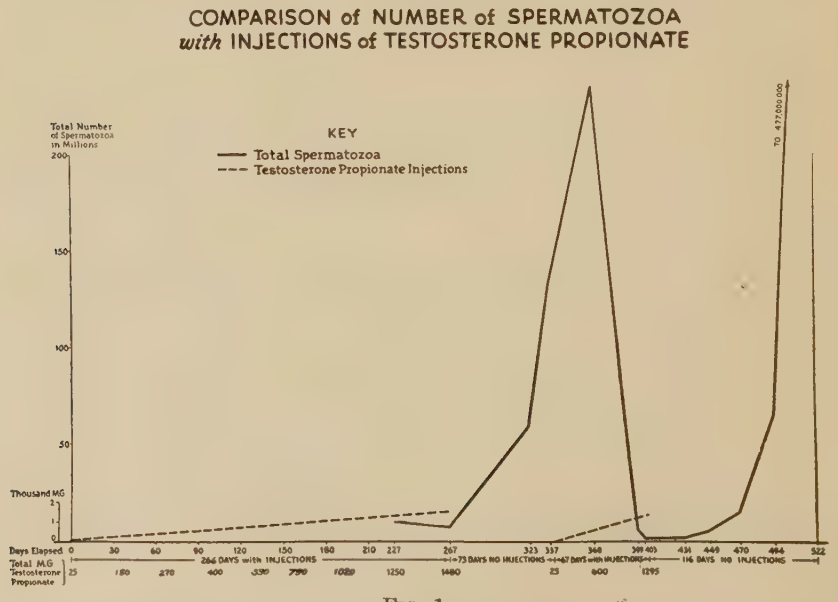


Fig. 1.

* The Schering Corporation kindly supplied the Oreton (testosterone propionate) used in this study.

genesis in the hypophysectomized rat.[1] It will also maintain, or repair damage to, the accessory reproductive organs of the castrated rat.[2] However, it does produce injury to the seminiferous tubules of young, growing, normal rats.[3]

The determinations of the number of spermatozoa in an ejaculation were obtained by counting them in a blood counting chamber (the diluent containing 5% soda and 1% formalin), multiplying the result by 1000 to obtain the number per cubic centimeter, and again multiplying the result by the number of centimeters of semen. The result plotted was the average of at least 2 to 4 determinations on each specimen.

Summary. The daily subcutaneous injection of 10 to 25 mg of testosterone depressed the spermatozoa count in a 67-year-old man. After its withdrawal the number of spermatozoa increased and during a second period of testosterone therapy the number of spermatozoa again decreased. The duration of the 2 periods of treatment, the daily dosage, and the variation in the spermatozoa counts are presented in the graph.

10528

Skin Transplantations Between Hairless and Haired Rats.*

HERBERT GERSHBERG. (Introduced by Ernst Fischer.)

From the Department of Zoology, University of Maryland.

The experiments reported here are an attempt to determine whether genetic hairlessness in the rat (inherited as a simple recessive) is due mainly to factors in the skin or to an endocrine abnormality. Emery[1] discusses a possible relationship between the hypophysis and the hairless condition. But Martin and Gardner,[2] feeding equivalent amounts of cysteine, cystine and glutathione to hairless rats, concluded that

1 Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Physiol.*, 1934, **107**, 508; Nelson, W. O., and Merckel, C., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 825.

2 Callow, R. K., and Deanesly, Ruth, *J. Biochem.*, 1935, **29**, 1424; Moore, C. R., and Price, D., *Endocrinology*, 1937, **21**, 313; Moore, C. R., and Price, D., *Anat. Rec.*, 1938, **71**, 59.

3 Moore, C. R., Lamar, Jule K., and Beck, Naomi, *J. A. M. A.*, 1938, **111**, 11; Moore, C. R., and Price, D., *Anat. Rec.*, 1938, **71**, 59.

* The author wishes to express thanks to Dr. S. O. Burhoe for his help and suggestions.

1 Emery, F. E., *Am. J. Physiol.*, 1935, **111**, 392.

2 Martin, G. J., and Gardner, R. E., *J. Biol. Chem.*, 1935, **111**, 193.

the mechanism for the hydrolysis of glutathione and other peptide linkages involving S-containing groups is hereditarily absent in the hairless rat, and that this faulty mechanism is responsible for the hairless condition.

The question still remains as to whether the mechanism resides in the follicles or elsewhere. Nor is the possibility of endocrine complication precluded. Skin transplantation offers a possible approach to the solution. Crew and Mirskaia[3] attempted transplantation between recessive hairless and haired mice with no success. David,[4] working with mice, obtained on a normal host one successful hairless graft, but this was replaced by host skin before any definite conclusions could be made. One normal graft on a hairless mouse regenerated hair 3 weeks after grafting and remained well haired until fixation, 126 days after.

At birth and up until about 19 days of age the hairless rats cannot be distinguished from their haired litter-mates. About 18-21 days after birth depilation begins and is usually complete in 2 weeks. The adult has subnormal vitality and a shorter life-span, and the female is generally sterile and non-lactating.

All the rats were operated on as soon after weaning age as possible, since any attempt to use younger rats met with failure.

In a preliminary series, pieces of skin were exchanged between 3 hairless and haired rats in the region dorsal to the scapula. The reactions were definitely negative, the grafts scabbing off within 2 weeks. Moreover, since Crew and Mirskaia, using dorsal transplants had obtained no takes, it was decided to try ventral transplants. The ventral skin was noticeably much thinner, and the reaction of the host to the graft was more favorable. The results reported here are all on ventral transplants.

A. *Autotransplants.* Several pieces of skin were cut off and sutured back in the same site. With the 2 haired rats thus operated on, an initial loss of hair was followed by a reappearance of hair on the graft in one case 16 days, and in the other, 25 days after operation. The 4 hairless autotransplants were successful, the grafts undergoing no noticeable absorption. But 18-30 days after operation hair growths appeared on the original hairless grafts. This growth consisted of several long, straggly hairs, similar to those found on hairless rats several months of age. In addition, such a secondary growth of hairs on the back and abdomen was noticed on these and almost all the hairless rats which had been operated on.

[3] Crew, F. A. E., and Mirskaia, L., *J. Genetics*, 1931, **25**, 17.

[4] David, L. T., *J. Exp. Zool.*, 1934, **68-69**, 501.

FIG. 1.
Hairless transplant on haired rat, 74 days after transplantation.

B. *Homoiotransplants.* Pieces of skin were exchanged between 6 hairless litter-mates. The reactions were antagonistic; the tissue was absorbed quickly or scabbed off. One graft "took" for 40 days and was then absorbed. On this graft a growth of several fine hairs occurred 23-26 days after operation.

Eighteen pieces of skin were exchanged between hairless and haired rats. Both series of grafts "took" equally well, but 2-4 weeks later most of the grafts in both series either scabbed or were absorbed. Therefore, although data on 25 rats are available, only 3 grafts can be called successful. Two are hairless transplants on haired rats, the other a haired transplant on a hairless rat.

Fig. 1 shows the successful hairless graft on haired rat No. 5,

74 days after operation. Twenty-five days after operation a few isolated fine hairs were noticed on the graft. These shed off after 10-12 days and were followed by a similar growth 14-17 days after. No subsequent hair growths occurred there after the second. The skin at this time showed the characteristics of hairless skin, being rough, thickened, and having a pinkish brown color. It became thicker with age and could very easily be identified by touch from the surrounding host skin. Observations on this graft were made up until the 80th day after operation, at which time the feeding experiment was begun.

On the hairless transplant on haired rat No. 344, (Fig.2) a growth

FIG. 2.
Hairless transplant on haired rat, 3 weeks after transplantation.

of several long hairs occurred 16-18 days after operation. This shed 8-10 days after. Unlike the other hairless transplant this one had not, after 9 months, undergone degeneration, but remained soft and pliable although hairless. Normal hairless skin of this age is considerably more thickened and wrinkled. Thus it seems that the effect of operation is to stimulate a few follicles to produce long hairs, but the hairless skin remains "hairless" even though in a "haired" environment.

The one successful haired graft on a hairless rat produced an abundant supply of hair. This growth began after 13 days and reached its peak about 2 weeks later. Forty-three days after operation the transplant underwent absorption which was complete within one week. In rats 21, 1296, 25, and 346 the second superficial layer scaling off the graft contained a new growth of numerous short hairs, the older hair having come off with the first layer.

The results of the transplants thus point to the conclusion that the factors for hairlessness reside principally in the skin. In the presence of so small a number of "takes", no conclusions as to the optimum age or sanguinity of host and donor can be ventured. However, the 3 "takes" were syngenesiotransplants; that is, donor or host were litter-mates. The operation was performed when the rats were comparatively young (19 days of age). Relative to this, a personal communication from Crew suggests the use of young animals. The most antagonistic reactions were encountered in the dorsal homoiotransplants in which the grafts sloughed off early as scabs. These reactions are very probably due to the thickness of the skin on the dorsum of the hairless rat, since no such sloughings were encountered in any of the ventral transplants.

C. *Stimulative Effect of Operation.* Crew observed a growth of fine hairs over the dorsum of hairless mice about 15 days after transplantation. David states that this growth was probably the periodic partial regeneration which naturally occurs in these mice. My experiments partially confirm Crew's, since my controls, the remainder of the hairless stock, have never shown any appreciable regeneration of hair. No difference in stimulative effect is manifested by the type of graft; *i. e.*, autotransplant or homoiotransplant, nor by the sex of the animal. These results, however, demonstrate that the operation itself produced such a regrowth of hairs.

Abdominal growth occurred before the dorsal growth. The amount of growth varied inversely with the age of the animal at the time of operation; only very little hair growth was observed in animals older than 80 days.

Fig. 3 shows an example of marked secondary growth, about a

FIG. 3.
Comparison between operated hairless rat with secondary hair growth and unoperated hairless rat of the same age.

week after the hair began to shed, in comparison with a control of approximately the same age. It should be mentioned that this secondary growth as stimulated by the operation skipped one hair cycle; thus, instead of occurring in the following period of hair growth it occurred in most of the cases in the second cycle after the operation.

The fact that this growth occurs not only at the site of operation but all over the body deserves further investigation.

Effect of Cysteine. The transplantations afford evidence that the hairless skin is the local expression of a gene complex. This seems difficult to connect with the results of Martin and Gardner unless we assume that the enzyme suggested by them is present in the skin of haired rats and not present in hairless skin. By feeding cysteine to the haired rat it was felt that it should be possible to reach a concentration in the blood by which the hairless transplant would be stimulated. The 2 rats with the hairless grafts were fed daily supplements of 50 mg of cysteine-HCl for 2 months, but there

was no increase in the hair on the graft. Two other rats, both hairless, one a litter-mate and one 111 days of age, were fed the same amount for the same length of time, but no change in the hair was apparent. The hairless skin of my rats may have been too old to be stimulated, since the youngest rat was 59 days old, whereas rats 30 days old were used by Martin and Gardner. However, Roberts,[5] using rats 28-42 days old, has repeated the work of Martin and Gardner and obtained no secondary growth on the hairless rats.

Summary. The results seem to indicate that the factors for hereditary hairlessness of the rat reside principally in the skin. The effect of operation on the hairless rat is to stimulate a secondary growth of hair, the amount of growth decreasing with the age of the rat. This growth is not confined to the site of operation but occurs over the whole body.

10529 P

Diagnosis of Pancreatic Disease; Limitations of Present Blood Diastase Test, Suggestion for Increasing its Effectiveness.

J. M. McCAUGHAN.

From the Department of Experimental Surgery, St. Louis University.

Although the diastatic activity of the blood was known as long ago as 1846[1] it was not until Wohlgemuth[2] developed his method of quantitative estimation that the subject began to receive the attention of clinicians. Since that time other more accurate methods have been devised for the micro-estimation of diastase in blood and urine.[3]

Clinical experience with the method in the diagnosis of pancreatic disease has led to the conclusion that it is one of the most valuable diagnostic procedures available particularly in acute pancreatitis.[4] Unfortunately, the value of the test is limited by the fact that the rise and subsequent fall in the concentration of the diastase after mechanical obstruction of the pancreatic ducts takes place over a comparatively short period of time, usually 2 to 3 days to attain a maximum height and 10 to 14 days to recede to normal.

With this restriction in mind, it is obvious that normal values

5 Roberts, E., *J. Biol. Chem.*, 1937, **118**, 627.

1 *Magendie Gaz. med. de Par.*, 1846, **1**, 73.

2 Wohlgemuth, J., *Bromchem. Z.*, 1908, **9**, 1.

3 Somogyi, M., *J. Biol. Chem.*, 1938, **125**, 399.

4 Cole, W. H., *Am. J. Surg.*, 1938, **40**, 245.

many times cannot be satisfactorily interpreted. Not only is this frequently true in the case of acute pancreatitis which may be seen by the clinician too late to obtain evidence of abnormal diastatic activity, but it is commonly the case, with such lesions as chronic pancreatitis, cysts and neoplasms where the diastase determinations are often of little or no value whatever.

Accordingly, the following experiments were carried out in an effort to overcome this inherent difficulty of the test. Dogs varying from 10 to 15 kg in weight were anesthetized with sodium-pentabarbital (nembutal) gr ½ per kg of body weight intravenously and local infiltration with 1% solution of novacain. A mid-line abdominal incision was made and a rubber balloon was introduced through an incision in the stomach wall and placed into the duodenum just past the pyloric ring. The balloon was then distended with water to a pressure of 90 mm of Hg. Oxalated blood samples were taken at intervals and the diastase concentration determined by the Somogyi method. At the onset of the experiments crude Secretin was given intravenously in doses of 0.5 cc per kg of body weight to provoke a good flow of juice.

In 2 experiments in which the balloon was distended, the diastase showed a rise of 250% in one hour in one and 380% in an hour and a half in the other. A control animal with the balloon left undistended showed no change in the diastase level after 2 hours; another control in which the common duct was ligated in order to exclude the possible influence of coincident biliary obstruction on the diastase level showed a rise of only 150% in 2 hours. The pancreatic ducts of a dog were then ligated as a preliminary procedure and time was allowed for development of atrophy and fibrosis in the gland. Then the experiment of occluding the duct openings by means of the distended balloon was repeated. No rise in the curve of the blood diastase was noted. The procedure had no harmful effect on the animals. Clinical application of these observations to the human has not yet been made, although we have carried out the introduction of the Miller Abbott tube and balloon into the duodenum under fluoroscopic control and have distended it without causing the patient distressing symptoms.

While it would be premature to forecast the clinical application of these observations, it may be suggested that an index to pancreatic function could be obtained in this manner. A normal pancreas might be expected to show a certain rise in the serum diastase after temporary obstruction by the balloon, whereas the serum diastase in cases of disease of the pancreas might remain unaltered or be changed but slightly.

10530 P

Anterior Pituitary Growth Factor and Blood Sugar.*

R. C. Weinstein. (Introduced by C. I. Reed.)

From the Department of Physiology, University of Illinois, College of Medicine.

That the growth acceleration induced by the growth factor of the anterior lobe of the pituitary body profoundly affects protein metabolism has been demonstrated by a number of workers. However, there appears to have been little attention given to a possible influence of *this particular factor* as such on carbohydrate metabolism. A survey of the literature by Van Dyke[1] does not contain any reference to a relationship between the growth factor and carbohydrate metabolism. Long[2] cited a few experiments in which a certain growth hormone preparation produced glucosuria but this preparation is obviously not the same as the one used in this investigation.

As an initial approach to this problem, the following group of experiments was undertaken.

Two dogs, weighing respectively 14 and 16 pounds, were fasted 12-15 hours. Blood samples were drawn and 50 units of Antuitrin Growth were injected intravenously. Blood samples were drawn at 1, 2, 24, and 48 hours. The results are shown in the chart (Fig. 1). Experiments were repeated at weekly intervals. The dogs were fed lightly after the 2-hour and 24-hour sampling, but each subsequent determination was made after 12-15 hours fast. Both dogs maintained approximately constant weight. Autopsy did not reveal any significant changes in any tissue.

Blood sugar was determined by the Somogyi modification of the Shaffer-Hartman method.

Both dogs became markedly depressed about 30 minutes after injections and after the first injection in each dog this depression was accompanied by violent sneezing which persisted for 15 to 20 minutes. This was attributed to an allergic reaction.

It will be noted in the two graphs that in both cases the fasting level was progressively lower with each succeeding experiment. The *exact* composition of Antuitrin Growth is not known, but according to assays by the manufacturers it contains negligible amounts of the

* Antuitrin Growth was made available by Parke, Davis and Company. This cooperation is gratefully acknowledged.

1 Van Dyke, H. B., *The Physiology and Pharmacology of the Pituitary Body*, University of Chicago Press, 1936.

2 Long, C. N. H., *Medicine*, 1937, **16**, 215.

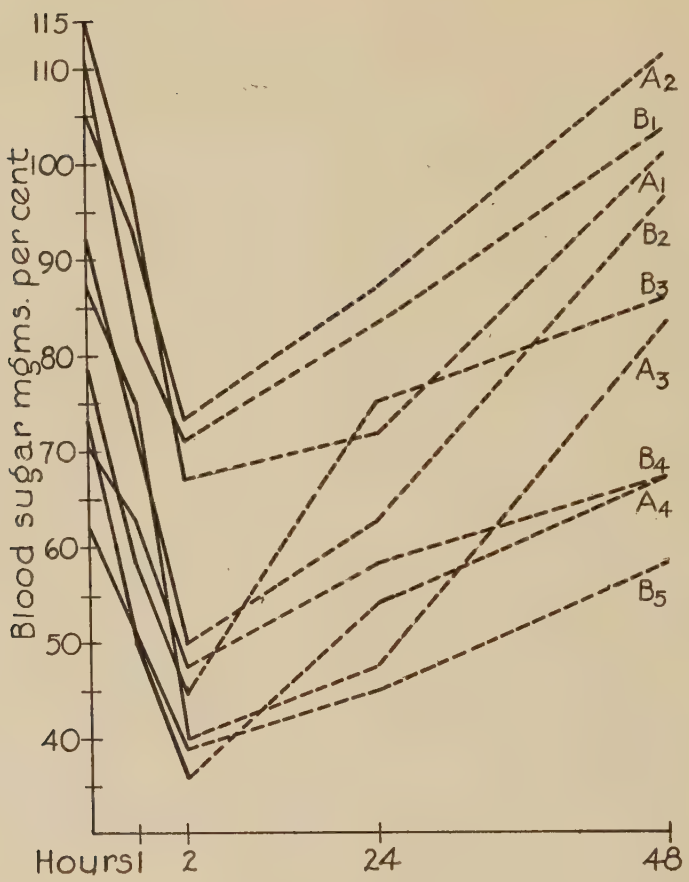


FIG. 1.

A—Male dog, 14 pounds, A_1, A_2, etc., blood sugar curve after 50 units growth hormone at successive weekly intervals.

B—Male dog, 16 pounds weight, B_1, B_2, same as above.

other anterior lobe factors that are more specifically related to carbohydrate metabolism. Also the solvent has no hypoglycemic action.

Summary. Intravenous injection of 50 units of Antuitrin Growth into adult healthy dogs induced progressive depression of fasting blood sugar for 2 hours. At 24 hours the level approached that found at 1 hour, and after a lapse of 48 hours the fasting level closely approximated the initial level.

10531

Free Volatile Acidity of Blood and Tissues Following Ingestion of Ethyl Alcohol.*

Theodore E. Friedemann and Thaddeus C. Kmieciak.

From the Laboratory of Chemical Bacteriology, Department of Medicine, University of Chicago.

Is the acidosis of alcoholic intoxication due to the accumulation of acetic acid as well as the production of abnormal quantities of lactic acid?[1] The presence of acetic has never been demonstrated. Analyses of tissues by means of the writer's recently published procedure for the determination of minute quantities of volatile acids[2] appeared to answer the question affirmatively. However, a subsequent study of the analytical method, as applied to tissues, has shown that a substantial part of the acid so determined is derived by hydrolysis of the "bound" volatile acids by the reagents during the steam distillation. By carrying out the steam distillation with a minimum of phosphoric acid and tungstate, it was found that normal tissues contain only traces of *free* volatile acid and that the latter are not apparently increased after ingestion of ethyl alcohol.

Analytical Procedure. The sample† is frozen and crushed by the method of Graeser, Ginsberg and Friedemann.[3] It is immediately swept into a small aluminum scoop; it is rapidly weighed. It is then transferred to the steam distillation flask, after which the scoop is again weighed. Analysis of 10 to 15 g samples is recommended. Fifteen cc of phosphoric acid solution,‡ 15 cc of 10% Na_2WO_4 and 10 cc of distilled water are then added. The flask is closed by means of a soft rubber stopper and the contents are mixed by violent shaking. The steam distillation and redistillation from acid-$MgSO_4$-HgO are carried out as already described. The dis-

* This study was aided by the Bartlett Memorial Fund of the University of Chicago.

1 Himwich, H. E., Nahum, L. H., Rakieten, N., Fazikas, J. F., DuBois, D., and Gildea, E. F., *J. Am. Med. Assn.*, 1933, **100**, 651.

2 Friedemann, T. E., *J. Biol. Chem.*, 1938, **123**, 161.

† In this laboratory all animals are anesthetized by means of pentobarbital sodium (Nembutal). Tissues are removed as soon as possible after the full effect of the anesthetic is noted. The anesthetic necessary for this work was donated by the Abbott Laboratories, North Chicago, Illinois.

3 Graeser, J. B., Ginsberg, J. E., and Friedemann, T. E., *J. Biol. Chem.*, 1934, **104**, 149.

‡ 55 cc of syrupy, 85% phosphoric acid are diluted to a volume of 1,000 cc.

tillate is aerated *15 minutes* by means of a rapid stream of CO_2-free air. Three drops of a 1% alcoholic solution of phenolphthalein are added. The acid is titrated by means of 0.01 *N* NaOH.

Free volatile acids of normal tissues. Representative data from 2 normal dogs are shown in Table I. The blanks in Expt. 1 required 0.57, 0.52, 0.58, 0.50 cc 0.01 *N* NaOH. The volatile acids from 10 to 15 g of tissue, with the exception of brain, required only a few drops more than the blank. The following are representative titrations of duplicate samples: 10 cc of blood required 0.60 and 0.58 cc of 0.01 *N* NaOH; 17.0 and 15.5 g of skeletal muscle, 0.59, 0.62; 16.5 and 15.5 g of liver, 0.70, 0.66; 17.0 and 15.5 g of kidney, 0.74, 0.90; 12.9 and 13.9 g of brain, 2.90, 2.90. Note that the variations in the titration of duplicates are within the errors of titration of the blanks. It should be noted further that these low results were obtained by the direct distillation of the tissue with steam. Very slightly lower results are obtained when protein-free filtrates are distilled.

Similar small yields of volatile acids have been obtained from tissues of other animals. The results vary slightly in each animal, but they agree, within the limit of error of the method.

In a few experiments, tissues were kept 30 minutes at room temperature before freezing in liquid nitrogen. An increase of volatile acidity was noted in most instances. In Exp. 3, for example, the volatile acidities of the immediately frozen samples and of the samples which were frozen after 30 minutes of incubation at room temperature, were as follows: skeletal muscle, 0.04, 0.07; heart muscle, 0.05, 0.24; liver, 0.08, 0.25; kidney, 0.24, 0.31; brain, 1.65, 2.01.

TABLE I.

Free Volatile Acids of Tissues.

Cc *N* or mM of free volatile acid per kg of tissue. The results also represent the cc of 0.01 *N* NaOH (minus the blank) required to titrate the volatile acids from 10 g of tissue.

	Normal animal		Normal animal after ethyl alcohol ingestion
	Exp. 1	Exp. 2	Exp. 3
Blood	0.05	0.12	0.05
Muscle, skeletal	.04	.04	.03
" heart	.11	.05	0
" stomach	.15	.11	.16
" diaphragm	.10		
Spleen	.03		
Liver	.09	.08	.15
Kidney	.17	.24	.19
Testicle	.08		
Brain	1.77	1.65	1.58

Free volatile acids following the ingestion of alcohol. 4.0 g of ethyl alcohol per kilo of body weight were given by stomach tube to a fasting normal male dog which weighed 29 kg; 150 cc of 96% "grain" alcohol were dissolved in 400 cc of water. One-half, or 200 cc of solution, was given at 8:45 A.M.; the other half was given at 9:20 A.M. Although the animal was greatly under the influence of the alcohol, it was conscious up to 10 A.M., at which time the anesthetic was given. The venous blood at this time contained 560 mg of alcohol per 100 cc. The results are shown in the last column of the table. The volatile acidity of all tissues, within the limit of experimental error, was the same as in the normal animals.

The writer's (unpublished) study of the volatile acids of tissues has shown that large quantities of these acids can be obtained by hydrolizing tissues with a 2 *N* H_2SO_4 for a period of about 6 hours. Acetic acid constitutes at least 95% of the volatile acids so obtained. It is not apparently obtained from the fat. The rate of liberation of this "bound" volatile acid parallels the rate of hydrolysis of the polysaccharide (total hydrolizable reducing substances-free sugar—glycogen). It is, therefore, most likely present in tissue as an acetyl radical attached to the polysaccharide; the polysaccharide, in turn, is combined with protein. The total volatile (acetic) acid content of tissues expressed as mM or cc *N* per kilo is approximately as follows: blood, 4 to 5; skeletal muscle, 4 to 5; liver, 6 to 8; kidney, 8 to 10; brain, 18 to 20. By comparing these data with those shown in the table, it is evident that the free acids constitute only about 1 to 3% of the total volatile acids of the tissue. Brain tissue is an exception; it apparently contains considerable quantities of free volatile acids and large quantities of easily hydrolyzed esters. Its content of total volatile acids also is unusually large.

The intermediary production of acetic acid and other volatile acids from the aerobic metabolism of carbohydrate and sugar metabolites, fats, and amino-acids by tissue slices in the Warburg apparatus has been abundantly demonstrated by the work of many investigators. Tissues, especially liver tissue, contain alcoholases.[4] The oxidation of alcohol by tissue slices, especially of liver, readily yields acetic acid.[5] Since these acids are formed in considerable quantity in the intermediary metabolism, why are they found free in such small concentration in tissue? The answer is that acetic acid is burned with great rapidity (author's unpublished data); it is removed as rapidly as it is formed in the intact normal animal. The maximum rate of

[4] Reichel, L., and Köhle, H., *Z. physiol. Chem.*, 1935, **236**, 158.

[5] Leloir, L. F., and Muñoz, J. M., *Biochem. J.*, 1938, **32**, 299.

oxidation of ethyl alcohol (about 4.5 mM per kilo per hour) is less than one-half that of the maximum rate of oxidation of sugar in the normal animal. The rate of acid production, assuming that all of the alcohol (4.5 mM) is converted into acetic acid, is relatively small and is well within the capacity of the animal to remove all of the acid by oxidation.

Summary. 1. A procedure is described for the determination of the free volatile acids of tissues. 2. Normal tissues of the dog, with the exception of brain, contain only traces, from 0 to 0.25 mM or cc *N*, of free volatile acidity per kg. This constitutes from 1 to 3% of the total volatile acids which can be obtained by hydrolysis with 2 *N* H_2SO_4. About 10%, from 1.5 to 2.0 mM, of all of the volatile acids of the brain are present either as free acid or in some form which is readily hydrolized. 3. The metabolism of ethyl alcohol in the dog does not result in an increase of the free volatile acids of the blood and tissues.

10532

Evidence of Local Protection Against Infection with Type I Pneumococcus.*

WILLIAM H. HARRIS.

From the Department of Pathology, School of Medicine, Tulane University, New Orleans.

The production of local protection or "local immunity" to microorganismal infection was first fully described by Wassermann and Citron[1] and interpreted by them as an *"Umstimmung"* or "retuning" of the local cells. This phenomenon has subsequently been observed but differently explained, especially by Besredka,[2] Gay,[3] and Opie.[4]

Bull and McKee[5] and recently Walsh and Cannon[6] demonstrated in rabbits a definite specific resistance of the upper respiratory tract to pneumococcal infection subsequent to specific local intranasal instilla-

* Aided by a grant from the David Trautman Schwartz Research Fund.

1 Wassermann, A., and Citron, J., *Z. f. Hyg. u. Infectionskr.*, 1905, **50**, 331.

2 Besredka, A., *Compt. Rend. Soc. de Biol.*, 1923, **88**, 1273.

3 Gay, F. P., *The Newer Knowledge of Bacteriology and Immunology*, Jordan, E. O., and Falk, J. S., University of Chicago Press, 1928, 881.

4 Opie, E. L., *J. Immunol.*, 1929, **17**, 329.

5 Bull, Carroll G., and McKee, C. M., *Am. J. Hyg.*, 1929, **9**, 490.

6 Walsh, T. E., and Cannon, P. R., *J. Immunol.*, 1936, **31**, 331.

tion. These observers point out that although the rabbit host seems protected in general, evidences of mouse-protective substances are not demonstrable. Gay and Rhodes,[7] on the other hand, found that only the derm was resistant to the streptococcus following experimental erysipelas whereas infection was produced by other routes of inoculation. Extensive literature in this field is afforded by Pacheco[8] and Gay.[9]

We employed both hairy and hairless white mice, and Type I pneumococcus A5-51R obtained through the courtesy of Dr. Kenneth L. Burdon and grown on blood-agar slants. When a saline suspension of the pneumococcus killed with 0.4% formalin was injected intraäbdominally on each of 5 days during a period of one week, the animals then survived for 5 to 6 days subsequent to an intraäbdominal injection of living pneumococci that was fatal to controls over night. In seeking a useful ratio of protecting factors to lethal dose it was found that .05 cc of saline suspension of a 24-hour growth, containing approximately one-half billion microorganisms per cc, killed mice within 24 to 48 hours when injected intraäbdominally, thus representing one minimal lethal dose. A similar dose injected subcutaneously killed in 36 to 72 hours. It was further ascertained that mice receiving 5 0.5 cc intraäbdominal injections during a week, of the formolized suspension containing approximately one billion per cc were protected practically 100% against an intraäbdominal injection of .05 cc of living cocci in suspension of about one-half billion per cc. With this standardization of protective procedure, the animals were grouped in various series representing similar routes of protective and lethal dosages and also divergences of these routes.

In Series 1, the routes of both injections, killed and living, were intraäbdominal; in Series 2, the protective injections were intraabdominal and the lethal doses were subcutaneous; in Series 3, both were subcutaneous; in Series 4, the protective injections were subcutaneous and the lethal were intraäbdominal.

Series 1: Ten mice received 5 intraäbdominal injections of the formolized suspension during one week, then .05 cc of the living suspension was administered intraäbdominally. All these animals survived.

Ten controls, 5 of which had been given 5 intraäbdominal injec-

7 Gay, F. P., and Rhodes, B., *J. Infect. Dis.*, 1922, **31**, 101.

8 Pacheco, G. A., *Arch. Path.*, 1932, **13**, 868.

9 Gay, F. P., and Associates, *Agents of Disease and Host Resistance*, Charles Thomas, Baltimore, 1935, 448.

tions of formolized saline solution, received .05 cc of the living suspension and all died within 24 to 48 hours.

Series 2: Five mice received 5 intraäbdominal injections of formolized suspension during one week, then .05 cc of the living suspension was administered subcutaneously. Four of the animals died within 4 days and one in 10 days.

Series 3: Five mice received 5 subcutaneous injections of formolized suspension. On the following day, .05 cc of the living suspension was given subcutaneously. The protective injections were closely grouped and contiguous, and the lethal dosage was injected within this area. All 5 were protected while 4 controls all died within 48 hours.

Series 4: Five mice received 5 subcutaneous injections of formolized suspension during one week. The following day .05 cc of the living suspension was given intraäbdominally. Four mice died within 3 to 5 days and one survived for 12 days.

Walsh and Cannon[10] found in rabbits that at least 5 daily nasal applications of the antigenic preparation were necessary for protection against the pneumococcal infecting dose. Barach[11] obtained some degree of protection in mice 3 days following one intraäbdominal protective dose, and demonstrated that it was type-specific. Inasmuch as the peritoneal cavity permitted of better retention of the stimulating or possibly desensitizing factor, the following tests were made:

Two mice received .5 cc of killed suspension intraäbdominally and, after 24 hours, were given the infecting dose by the same route. Both animals survived.

Two mice received a similar protecting injection and the infecting dose at the same time. Both died in 48 hours.

Two controls received the identical infective dose and both died in 48 hours.

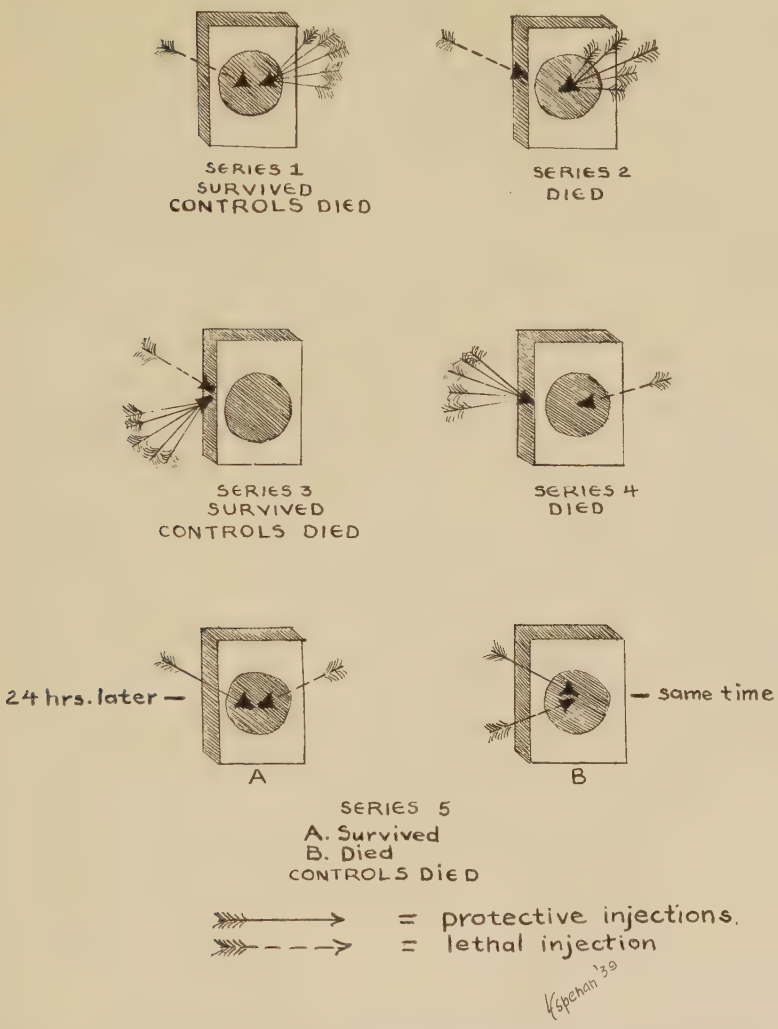
These experiments have been confined to the subject of local protection as contrasted with systemic immunity. No tests of specificity have been carried out.

Duration of protection of the 10 surviving mice in Series 1. Groups of these were given another injection of living pneumococci after 1, 2, 4, and 6 weeks. At 6 weeks, the resistance had abated inasmuch as the animals died after from 4 to 6 days, whereas controls died within 48 hours. A few tests of the peritoneal fluid of such

[10] Walsh, T. E., and Cannon, P. R., *J. Immunol.*, 1936, **31**, 331.

[11] Barach, A. L., *J. Exp. Med.*, 1928, **48**, 83.

GRAPH I



protected animals have failed to show capsular swelling or bactericidal properties.

From these results it is concluded that local protection against Type I pneumococcus is producible in the white mouse whereas infection and death occur from injections administered in untreated areas, thus indicating absence of, or at least deficient, systemic protection.

The duration of this protection extends from 4 to 6 weeks.

The protection of mice with one injection of the formolized suspension just 24 hours prior to the administration of an infecting

dose that kills controls within 48 hours, would appear to question seriously the formation of active immune factors, either humoral or cellular. It is suggested that the "shock-tissue" of the particular area is desensitized to the living pneumococcus by the primary injection of the formolized suspension.

It seems likely that with the recent interest awakened in this field that practical application will be attempted especially in the specific prophylaxis of those diseases gaining access through the upper respiratory tract, notably meningococcal meningitis and anterior poliomyelitis.

10533

A Simple Method for Accurate Injection of Small Volumes of Fluid.

GEORGE E. BURCH.

From the Department of Medicine, School of Medicine, Tulane University of Louisiana, New Orleans, La.

In order that the volume of dye, patent blue V, injected intradermally during studies on the linear rate of lymph flow in the superficial lymphatics of the skin of man by the McMaster[1] method, might be constant and accurate, an attachment was constructed to fit an ordinary Becton-Dickinson tuberculin syringe. The piece of apparatus proved to be simple, accurate, and very useful for these studies, and especially advantageous for colored solutions which preclude the reading of the scale on the cylinder of a syringe. Since it might prove useful to others whenever very small volumes of fluid are to be accurately measured and delivered with constancy into the tissues of the organism, the apparatus was considered worthy of report.

The attachment consists essentially of 3 parts (Fig. 1) which are mounted and used as follows: The cylinder of a tuberculin syringe is slipped into part *e*, which is fixed to the lip of the cylinder by screwing part *b* into *e*. Part *a* is then screwed on part *e*. The plunger is then inserted through the central openings in parts *a*, *b* and *e* and into the cylinder of the syringe until its head rests against *a*. The outer circumference of *a* was marked off in equal parts so that with the aid of a pointer *d*, fixed on *e* by screws, whole and fractions of a turn of *a* could be determined. The threading on *a* and *e* is such that one com-

[1] McMaster, P. D., *J. Exp. Med.*, 1937, **65**, 347.

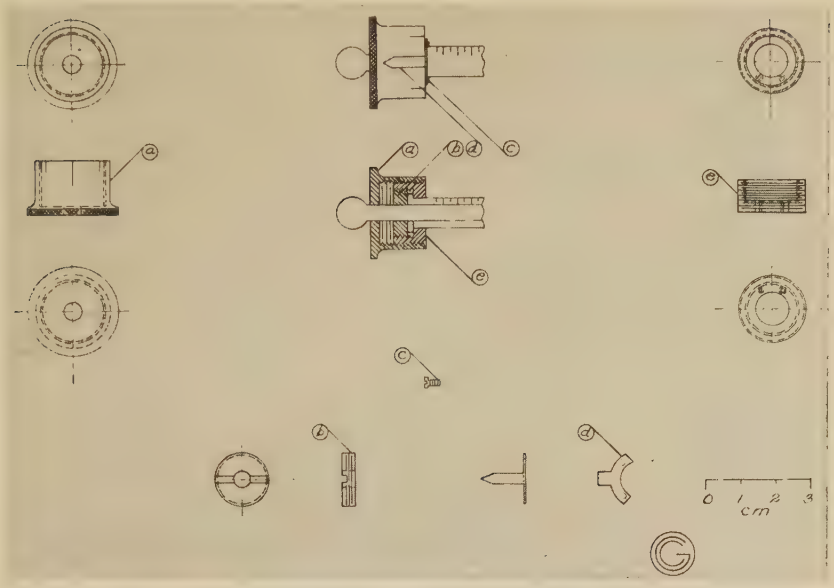


FIG. 1.
Drawing illustrating the construction of the apparatus.

plete turn of *a* on *e* will permit the plunger to be pushed farther into the cylinder and deliver approximately 0.01 cc of fluid before its head again comes to rest against *a*. Fractions of a turn of *a* will permit the delivery of fractions of 0.01 cc of fluid. The apparatus, mounted for use, is shown in Fig. 2. The attachment is made entirely of brass and can be sterilized, mounted in place on the syringe, by autoclaving or boiling.

The apparatus was calibrated as follows: Part *a* was unscrewed as far back on part *e* as possible and then mercury was drawn into the needle and syringe. After carefully removing all bubbles of air, the plunger of the syringe was pushed into the cylinder until the head of the plunger rested against part *a*. Then *a* was screwed one complete turn on *e* and the plunger was pushed into the cylinder of the syringe

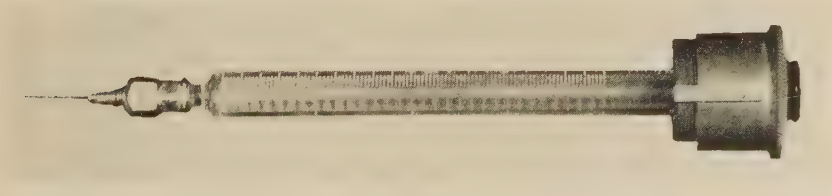


FIG. 2.
The apparatus ready for use.

TABLE I.
Results of Calibration of the Apparatus by Delivering and Weighing Mercury Following a Complete Turn of part *a* on *e*.

Successive turns of part *a* on part *e*	Wt of mercury delivered, g	Volume of mercury delivered, cc	Variations from the mean, cc	% variation from the mean
1st	.1211	.0090	—.0003	3.23
2nd	.1244	.0092	—.0001	1.06
3rd	.1242	.0092	—.0001	1.06
4th	.1251	.0093	.0000	0.00
5th	.1255	.0093	.0000	0.00
6th	.1261	.0093	.0000	0.00
7th	.1271	.0094	+.0001	1.06
8th	.1268	.0094	+.0001	1.06
9th	.1280	.0095	+.0002	2.15
10th	.1227	.0091	—.0002	2.15
Mean	.1245	.0093		
Minimum	.1211	.0090		
Maximum	.1280	.0095		

until the head of the plunger again rested on *a*, ejecting the mercury on a scale to be weighed and its volume determined. This process was repeated until *a* was screwed as far as possible on *e*. This procedure permitted 10 full turns of *a* on *e*, or 10 separate ejections of mercury. The results of the calibration are shown in Table I. It can be seen from the table that one complete turn of *a* permits the delivery of 0.0093 cc $\pm$ 0.0003 cc. The maximum variation encountered was 3.2%, which is relatively small for the volumes of fluid ejected. With a specially constructed syringe this error could be further reduced. Desired fractions of 0.0093 cc of fluid can be accurately delivered by making the necessary fractions of a turn of *a* on *e*.

Although the above described apparatus permits the delivery of a total volume of 0.10 cc without refilling the syringe, this could be increased by increasing the length of parts *a* and *e*.

Summary. A simple piece of apparatus for an accurate and constant injection of small volumes of fluid into the tissues of man is described. It is especially useful when colored solutions, which preclude the reading of the scale on a syringe, are used.

10534 P

Effect of Pseudopregnancy on the Life-Span of Adrenalectomized Cats.

W. D. Collings. (Introduced by W. W. Swingle.)

From the Biological Laboratory, Princeton University, Princeton, N. J.

Rogoff and Stewart observed that pregnancy and pseudopregnancy would greatly extend the survival periods of bilaterally adrenalectomized dogs.[1, 2] This observation has since been confirmed,[3] and more recently it has been shown that when pseudopregnancy is experimentally induced, it is just as effective in alleviating the symptoms of adrenal insufficiency as spontaneous pseudopregnancy.[4, 5] Experiments on the adrenalectomized, pseudopregnant ferret indicate that it is possible to obtain survivals which are, on the average, 5 times as long as those of the anestrus or male controls.[6]

In the experiments reported here, the right adrenal glands were removed from 10 mature female cats. Nine of these animals were driven into estrus by injections of a menopause urine extract (Gamone*), while the tenth animal came into spontaneous estrus after the operation. Gamone has been found effective in producing estrus and mating reaction in the dog,[7] but for use in the cat it was necessary to run a series of preliminary tests to determine the proper dosage. The results of these experiments indicated that 20 R.U. per kilo body weight per day would produce cornified vaginal smears and typical mating reactions after an injection period of 4 to 6 days (Table I). During the estrus period the cats were mated with a normal male, and in some cases they were greatly stimulated with the glass rod while taking vaginal smears. Thus every attempt was made to secure luteinization of the estrous ovaries by each animal's own pituitary gland secretion.

Immediately at the end of the mating period, the remaining adrenal gland was removed with as much care as possible in order to

1 Rogoff, J. M., and Stewart, G. N., *Am. J. Physiol.*, 1927, **79**, 508.

2 Rogoff, J. M., and Stewart, G. N., *Am. J. Physiol.*, 1928, **86**, 20.

3 Pfiffner, J. J., Swingle, W. W., and Vars, H. M., *J. Biol. Chem.*, 1934, **104**, 701.

4 Swingle, W. W., Parkins, W. M., Taylor, A. R., and Morrell, J. A., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 94.

5 Swingle, W. W., Parkins, W. M., Taylor, A. R., Hays, H. W., and Morrell, J. A., *Am. J. Physiol.*, 1937, **119**, 675.

6 Gaunt, R., and Hays, H. W., *Am. J. Physiol.*, 1938, **124**, 767.

* Through the courtesy of Dr. J. A. Morrell of E. R. Squibb and Sons.

7 Leathem, J. H., *Endocrinol.*, 1938, **22**, 559.

TABLE I.
Data on Pregnant and Pseudopregnant Adrenalectomized Cats.

Cat	Wt, kg	Period injected, days	Total R.U. injected	Days estrus	No. times mated	Interval between operations, days	Survival period, days*
1	3.2	5	320	5	8	64	20
2	2.9	5	290	6	5	18	26
3	3.1	5	305	6	1	18	35
4	3.3	4	262	6	13	17	22
5	2.2	6	270	5	10	28	21
6	3.0	5	280	6	14	21	28
7	2.3	Spontaneous estrus		6	13	47	20
8	2.9	4	240	5	5	20	14
9	3.2	4	240	4	3	20	24
10	3.2	4	256	5	7	18	22

*Average survival 23.2 days.

minimize the chances of shock affecting the survival time. No cortical hormone was administered, except to cat 1, which received injections for 2 days following the second operation. She survived 20 days after the hormone was withdrawn (Table I).

The survival periods listed in the table indicate that only 1 animal failed to survive as long as 20 days, and that the average for 10 animals was 23.2 days. In contrast to this figure, the average survival period was found, by Swingle and Pfiffner[8] in this laboratory, to be 8.15 days for 138 untreated male and female cats.

Upon autopsy animals 1, 7, 9 and 10 were found to be pregnant. Although the number of pregnant cases is small, it seems likely that the cat and dog do not differ with respect to the extended life-span of pregnant animals after bilateral adrenalectomy. Apparently the difficulties of demonstrating the lengthened survival are greater in the cat. In this connection, previous workers[9, 10] have been unable to demonstrate extensive survival in cats from which both adrenals were removed after pregnancy had been diagnosed.

Sections of the ovaries and uteri of the 6 non-pregnant animals indicated that they were in a regressing pseudopregnant condition. This was most obvious in the tissues of the 28-day and 35-day animals. The number and complexity of the endometrial glands were still great in most cases, and some of the ovarian sections were almost a solid mass of compact luteal tissue. A few of the ovaries exhibited lutein cysts which were attributed to a possible over-dosage of Gamone. These were not found in cats which had survived beyond

[8] Swingle, W. W., and Pfiffner, J. J., *Medicine*, 1932, **11**, 371.

[9] Rogoff, J. M., and Stewart, G. N., *Am. J. Physiol.*, 1929, **88**, 162.

[10] Corey, E. L., *Physiol. Zool.*, 1928, **1**, 147.

24 days. The pseudopregnant condition in the cat has previously been studied in great detail.[11, 12] When it is produced entirely by hormone injections,[11] the length of the period is 40-44 days. On the other hand, pseudopregnancy as the result of a sterile mating is said to end in 33 days.[12] In any case, the luteal phase, during which one may expect survival of the female from adrenal insufficiency, probably lasts about 35 days. It is likely that this figure represents the maximum survival period obtainable under these conditions.

The hormone of the corpus luteum, secreted during the luteal phase of the ovary, is capable of ameliorating the symptoms of adrenal insufficiency. This is in agreement with work on the ferret and the rat[6, 13, 14] in which it has been shown that it is possible to prevent the symptoms of adrenal insufficiency by administration of the proper dosage of pure crystalline progesterone. It should be equally feasible to demonstrate the efficacy of the pure hormone in lengthening the life-span of the adrenalectomized cat.

10535

Negative Effect of Gastric Juice Administered Intravenously to Patients with Pernicious Anemia.

R. W. HEINLE AND F. R. MILLER.

From the Department of Medicine, Western Reserve University School of Medicine, and the Lakeside Hospital, Cleveland.

Evidence has been produced[1] to show that the site of interaction between intrinsic (gastric) factor and extrinsic (food) factor is in the intestinal tract and not parenterally. It is also true that concentrated gastric juice when administered intramuscularly will produce a hematologic response in a patient with pernicious anemia.[2] It appears from the nature of the response that the stimulus is specific and that concentrated gastric juice does contain a hematopoietic principle

11 Foster, M. A., and Hisaw, F. L., *Anat. Rec.*, 1935, **62**, 75.

12 Gros, G., *C. R. Soc. de Biol.*, 1935, **118**, 1575.

13 Greene, R. R., Wells, J. A., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 83.

14 Fischer, A., and Engle, M., *Lancet*, 1939, **236**, Feb. 11, 354.

1 Castle, W. B., Heath, C. W., Strauss, M. B., and Heinle, R. W., *Am. J. Med. Sci.*, 1937, **194**, 618.

2 Morris, R. S., Schiff, L., Burger, G., and Sherman, J. E., *Am. J. Med. Sci.*, 1932, **184**, 778.

effective in pernicious anemia when administered intramuscularly.

If the interaction between the intrinsic and extrinsic factors can take place parenterally, and if the intrinsic and extrinsic factors can be absorbed as such from the intestinal tract in the normal individual, it would seem possible that administration of normal gastric juice intravenously to a patient with pernicious anemia might be capable of producing a hematologic response. This experiment, therefore, was performed on 2 patients.

The gastric juice was obtained from a normal, healthy individual in a fasting state after administration of 0.5 mg of histamine. The gastric juice was immediately neutralized with 5% sodium hydroxide and passed through a Berkefeld V filter. This latter procedure does not decrease the effectiveness of the gastric juice.[3] It was stored in an icebox until used but no gastric juice older than 4 days was employed.

Patient one received intravenously, over a period of 6 days, 190 cc of this whole, neutralized gastric juice which had been passed through a Berkefeld V filter. His diet contained 70 g of protein daily. There was no reticulocyte response in 10 days and no increase in red blood corpuscles or hemoglobin. There was subsequently a good response to liver extract* given intramuscularly (Table I).

Patient two received intravenously, over a period of 6 days, 175 cc of whole, neutralized gastric juice which had been passed through a Berkefeld V filter. His diet contained 150 g of protein daily. There was no reticulocyte response in 10 days and no increase in red blood corpuscles or hemoglobin. Subsequently, there was a good response to liver extract* administered intramuscularly (Table I).

Negative results with whole, neutralized gastric juice which had been passed through a Berkefeld V filter and administered intravenously to patients with pernicious anemia indicate either: (a) That there is no extrinsic factor as such present in the blood stream or in other parenteral sites to enable a reaction to take place; or (b) that the environment is not suitable for such a reaction; or (c) that insufficient quantities of intrinsic factor were introduced. Taylor, *et al.*,[3] have shown that the optimum pH for interaction between intrinsic and extrinsic factor is between pH 7.0 and 8.0 so that the pH of most of the parenteral tissues would constitute a favorable environment. Also, it is known[4] that liver extract, when injected parenterally, is 50

* Solution Liver Extract, Concentrated, Lilly, 2 units per cc.

3 Taylor, F. H. L., Castle, W. B., Heinle, R. W., and Adams, M. A., *J. Clin. Invest.*, 1938, **17**, 335.

4 Strauss, M. B., Taylor, F. H. L., and Castle, W. B., *J. Am. Med. Assn.*, 1931, **97**, 313.

TABLE I.
Negative Effect of Gastric Juice Administered Intravenously.

	Patient 1				Patient 2			
Day	RBC	Hgb* %	Retic. %	Therapy	RBC	Hgb* %	Retic. %	Therapy
				cc gastric juice intravenously				cc gastric juice intravenously
0	1.60	38	1.8	25	1.61	43	0.8	25
1	1.62	43	1.3	50	1.95	55	0.3	30
2	—	—	—	25	1.93	47	0.7	30
3	1.62	46	0.6	30	—	—	1.2	30
4	1.54	43	0.6	30	1.54	42	1.1	30
5	1.56	40	0.4	30	1.44	41	0.8	30
6	1.52	44	0.2	—	1.58	42	0.6	—
7	1.81	45	0.2	—	1.54	40	0.8	—
8	1.55	41	0.3	—	1.57	46	0.6	—
				cc liver ext. intramusc.				cc liver ext. intramusc.
9	—	—	0.2	—	1.55	44	0.6	10
10	1.50	41	0.7	10				
1	1.78	44	0.9	5	—	—	1.2	—
2	1.82	47	1.1	5	1.68	46	1.3	—
3	1.82	48	2.5	5	1.68	45	3.8	5
4	1.72	48	13.1	5	1.89	45	8.1	5
5	—	—	—	5	1.89	48	13.6	5
6	—	—	—	5	1.67	48	12.8	5
7	—	—	—	5	2.13	57	9.4	5
8	—	—	—	5	—	—	—	5
9	—	—	—	5	2.55	62	4.2	5
10	1.70	46	0.8	5				
15	2.19	55	0.9					

*100% Hgb = 15.6 g per 100 cc.

to 60 times as effective as the same dose given orally. If the same reasoning could be applied to intrinsic factor, it would appear that enough gastric juice had been administered to these patients since 75 cc of gastric juice administered daily by mouth with beef muscle is effective in producing a response in patients with pernicious anemia.[5] Thus, the most reasonable conclusion is that there is no extrinsic factor as such present in the blood or elsewhere parenterally to provide a substrate for interaction with the intrinsic factor present in the gastric juice administered intravenously.

The negative results obtained here also suggest that the positive effect of concentrated gastric juice injected intramuscularly[2] may be due to *in vitro* formation of a material similar to liver extract or some precursor of liver extract and not to intrinsic factor alone.

Conclusion. Normal, whole, neutralized gastric juice which had

[5] Castle, W. B., and Townsend, W. C., *Am. J. Med. Sci.*, 1929, **178**, 764.

been passed through a Berkefeld V filter and administered intravenously to patients with pernicious anemia does not produce a hematologic response probably because there is no extrinsic factor as such in the parenteral tissues to provide a substrate for interaction.

10536 P

A Pneumonia Virus of Swiss Mice.

FRANK L. HORSFALL, JR., AND RICHARD G. HAHN. (Introduced by J. H. Bauer.)

From the Laboratories of the International Health Division of The Rockefeller Foundation, New York.

A virus capable of inducing fatal pneumonia in Swiss mice has been isolated from normal mouse lungs, and its immunological characteristics are now being studied in detail.

Twenty-one groups of normal Swiss mice were inoculated intranasally under ether anesthesia with 0.05 cc of lung-suspensions from uninoculated mice. Serial mouse-passage was carried on with each group, using 10% to 30% lung-suspensions. Passages were made at an average interval of 7 days; usually 6 mice were used in each group. The mice were obtained from 6 different breeders. Initially, passages were made without regard to the breeder from whom the mice had been obtained. Lately, however, passages have been made in mice from each individual breeder in order to determine the source of the virus. Definite areas of pulmonary consolidation were present in 43% of the groups in the third serial passage, and in 52% of the groups at the sixth passage. Death occurred as early as the fourth passage, and by the sixth passage deaths were recorded in 24% of the groups. Cultures of the mouse-lung suspensions were made routinely and were sterile in a great majority of instances. Rabbits were injected intraäbdominally with virus-containing material from various passages. They were bled before injection and again 8 to 10 days afterwards. Their serum was tested for the presence of antibodies capable of neutralizing the various strains of virus in the manner described by Magill and Francis.[1]

Fatal pneumonia was caused by 0.05 cc of a 10^{-3} to 10^{-4} dilution of infected mouse lung, and definite pulmonary consolidation was pro-

[1] Magill, T. P., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 463.

duced by 10^{-5} to 10^{-6} dilutions. The virus was filtrable through Berkefeld V and N candles, passed through graded collodion membranes[2] with an APD of 300 mμ, and was retained by a Seitz filter. Centrifugation for 1 hour at 13,000 rpm did not cause complete sedimentation of the virus. Virulence decreased very rapidly at room temperature, and in broth suspensions as much as 99% disappeared in 1 hour. Heating to 56°C for 30 minutes inactivated the virus. It has been preserved at –80°C and by drying in the frozen state. The virus has been carried in chick-embryo-Tyrode tissue-culture medium[3] through 10 successive transfers, although with considerable reduction in virulence. The virus was strictly pneumotropic for mice. The susceptibility of mice from various breeders showed marked differences.

Microscopically the pulmonary lesions showed dense peribronchial and perivascular accumulations of mononuclear cells with hyperemia and edema. The bronchial epithelium was well preserved and sometimes appeared hyperplastic. The intracerebral, intraabdominal, intravenous, or subcutaneous injection of the virus in mice produced no recognizable lesions. Neither the brains nor the livers of mice injected intracerebrally and intraäbdominally, respectively, showed the presence of the virus. Ferrets did not develop symptoms, fever, or pulmonary lesions when inoculated intranasally, nor did the virus become adapted to ferrets on serial passage. However, the virus could be recovered from the lungs of inoculated ferrets. Ferrets that received the virus intranasally were fully susceptible subsequently to the PR8 strain of influenza-virus. Mice actively immunized by 2 intraäbdominal injections of infected mouse lung were immune to intranasal inoculation of the virus, but were not immune to 10 lethal doses of PR8. Conversely, mice actively immunized in a similar manner with PR8 were not immune to 10 lethal doses of this pneumonia-virus of mice. Rabbits injected intraabdominally with 3.0 cc of either tissue-culture supernate or 10% saline suspension of infected mouse lung produced antibodies against the virus. Eleven strains have been isolated and antisera have been prepared against 6 of these. Cross-neutralization tests have shown these strains to be immunologically identical. Dochez, Mills, and Mulliken[4] and Gordon, Freeman, and Clampit[5] found viruses in the

[2] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

[3] Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.

[4] Dochez, A. R., Mills, K. C., and Mulliken, B., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 683.

[5] Gordon, F. B., Freeman, G., and Clampit, J. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **39**, 450.

lungs of normal mice, but were unable to produce antisera against these agents.

Serum from laboratory workers in contact with mice and also from some human beings who have had no contact with mice neutralized 500 lethal doses of the virus. The serum of normal mice from certain breeders was also capable of neutralizing the virus. Cross-neutralization tests with numerous antisera against ferret, mouse, and tissue-culture strains of epidemic-influenza virus and against mouse and tissue-culture strains of this pneumonia-virus of mice have been difficult to assess. With certain of these antisera reciprocal cross-neutralization has been observed when 1 :2 dilutions of the sera were tested. The evidence available suggests the presence of a common minor antigenic component in both viruses.

10537

Black Widow Antivenin Production in Rabbits.

DONN SMITH AND FRED E. D'AMOUR.

From the Biologic Research Laboratory, University of Denver.

Antiserum against the venom of the black widow spider has been successfully produced in sheep.* The size of this animal makes it necessary to use large numbers of spiders, about 3,000 per sheep over a 6 months' period. The death of an immunized animal consequently represents a considerable loss. In view of the ability of the rabbit to produce highly potent antipneumococcal sera we felt it worth while to investigate this animal's possibilities as a producer of antivenin.

Spiders were collected in the vicinity of Denver and the glands removed by the method previously described.[1] The venom-glands were macerated in saline, the debris removed by filtration through cotton and injections made subcutaneously. Three adult male rabbits, weighing approximately 4 kg, were immunized as follows: Injections were made every other day; for the first 2 weeks one-fourth the venom in one spider was given; for the next 2 weeks, one spider per injection; for the next 2 weeks, 2 spiders per injection and for the last 5 weeks 8 spiders per injection.

Tests for neutralizing power were made by adding varying amounts

* Anti-Black Widow Spider Serum, Squibbs.

1 D'Amour, F. E., Becker, F. E., and Van Riper, W., *Quart. Rev. Biol.*, 1936, **11**, 123.

of serum to solutions containing known amounts of venom, permitting the solutions to stand overnight, and then injecting intraäbdominally, into young rats weighing about 50 g. Control solutions of venom, unneutralized, were injected into litter-mates.

Results. Serum obtained at the end of the 5th week showed little if any neutralizing power. Table I shows the results obtained after 11 weeks' immunization, each rabbit having received the venom from approximately 150 spiders. For comparison, results previously obtained[2] with sheep serum are given. The sheep serum was obtained after about 6 months of immunization, some 3,000 spiders having been used per sheep. The average lethal dose (A.L.D.), that is, the amount of venom necessary to kill 50% of the rats, was determined at each assay and was one-fourth the amount of venom contained in 1 spider.

TABLE I.

No. of Rats	Material Injected per Rat	% Surviving
4	2 A.L.D. venom plus 1 cc non-immune serum	0
4	4 " " " .1 " serum (best rabbit)	50
4	2 " " " .1 " " " "	100
4	4 " " " .1 " " (poorest rabbit)	0
4	2 " " " .1 " " " "	100
10	10 " " " .1 " sheep serum	30
10	5 " " " .1 " " "	50

Conclusions. Rabbits produce black-widow antivenin which, taking into account the shorter injection period and the relatively small number of spiders necessary, compares reasonably well with that produced in the sheep. This finding may be of importance in the production of antivenin commercially, where only small amounts of readily available serum are desired.

2 D'Amour, F. E., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 262.

10538

A Simple Accurate Method for Extraction of Estrogenic Substances from Human Urine for Bioassay.*

THOMAS H. CHERRY AND MILTON J. BERNSTEIN.§ (Introduced by W. J. MacNeal.)

From the Endocrine Research Laboratory, Department of Gynecology, New York Post Graduate Medical School and Hospital, Columbia University.

In the last few years there have been published a number of methods for the extraction of estrogenic substances from human urine. The methods of Laqueur, Koch, and Kurzrok[1, 2, 3] have been found most satisfactory by this laboratory. Unfortunately the method of Cohen and Marrian[4] has not as yet been made applicable to the determination of estrin in the urine of the non-pregnant female. There is one main disadvantage in the first 3 named methods, and that is the length of time required for the entire extraction process. It would evidently be to great advantage to devise a method which would be simple in procedure, rapid and as satisfactory as the methods of Laqueur, Koch, or Kurzrok. Therefore we present the results of our experimental work.

It is known from the researches of Cohen and Marrian, Zondek[4, 5, 6] and others that a certain portion of the estrogenic substances present in the urine exists in a combined form. In this form it is either biologically inert or non-extractable. This is overcome to a great extent by acid hydrolysis of the specimen. The urine is acidified with 50 cc concentrated hydrochloric acid (35-37%) per liter of urine and then boiled for a period of one hour. Among other workers there has been a considerable difference of opinion as to the amount of acid to use and the length of time the specimen should be heated in the hydrolytic process. Smith and Smith[7] claim to have obtained their maximum increase in potency after boiling 2 hours with 15 vol. % HCl and 4% Zn; whereas Browne, *et al.*,[8] claim that if more than 10 cc concentrated HCl is used per liter of urine, there is de-

* This research was supported by a grant from the Alice L. Filbert Fund.

§ Present address, Endo Products, New York City.

1 Borchardt, Dingemanse, and Laqueur, *Naturwissenschaft*, 1934, **22**, 190.

2 Gallagher, Koch, and Dorfman, PROC. SOC. EXP. BIOL. AND MED., 1936, **33**, 440.

3 Kurzrok and Ratner, *Am. J. Obst. and Gyn.*, 1932, **23**, 689.

4 Cohen and Marrian, *Biochem. J.*, 1934, **28**, 1603.

5 Zondek, B., *Nature*, 1934, **133**, 209.

6 Zondek, B., *Ark. Kemi., Min. O. Geol.*, 1934, part 3, paper 24.

7 Smith, G. V., and Smith, O. W., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 460.

8 Venning, Evelyn, Harkness, and Browne, *J. Biol. Chem.*, 1937, **120**, 225.

struction of estrin. Our experimental work has indicated that the actual method of extraction is a factor whose importance has been insufficiently stressed in the past. Using our method of extraction the results did not show an increase in potency by boiling the urine more than one hour. Some specimens were boiled for periods as long as 4 hours with no definite increase in yield as compared to a control specimen which was boiled for only one hour. However, it was found that boiling the urine for a period of less than one hour did show a definite decrease in potency when tested biologically. If less than 50 cc concentrated HCl was used a longer period of boiling was necessary in order to obtain the same yield as the control specimen which had been boiled with 50 cc of acid. The addition of more than 50 cc HCl per liter did not increase the yield to any noticeable extent.

In order to extract the estrin after hydrolysis, the urine is cooled and shaken vigorously on a mechanical shaker with an equal volume of ethyl ether (U.S.P.) for a period of one hour. This volume of ether and the shaking time were decided upon after numerous extractions had been made with different volumes of ether and different shaking times. For this extraction a "Camp" shaker was used which gives up to 240 circular shakes per minute. It can also be seen from Table I that reëxtraction with ether of the hydrolized urine by shaking one hour yielded no further estrogen.

The emulsion of ether and urine is now put into a separatory funnel and to this mixture is added 30-50 cc sodium taurocholate† (10% sol.) which serves to break up the emulsion. The aqueous layer is discarded and to the ether fraction is added 6 cc sesame oil. The ether is evaporated on a hot water bath. This leaves an oily extract which is ready for bioassay.

In order to determine the accuracy of this extraction process, the

TABLE I.

Extract No.	Process	Yield/Liter R.U.	Yield/Liter I.U.
1	Boil 1 hr, shake 1 hr	40	200
1a	Reextraction of No. 1	0	0
2	Boil 1 hr, shake 1 hr	42.8	214
2a	Reextraction of No. 2	0	0
3	Boil 1 hr, shake 1 hr	37.5	187+
3a	Reextraction of No. 3	0	0
4	Shake 1 hr only	8+	40+

The extractions were carried out on pooled specimen No. 1.

† This substance has been biologically assayed, and does not show any estrogenic activity.

extraction methods of Laqueur, Koch, and Kurzrok were used as comparisons as they are the methods most used in endocrine laboratories.

From 14 to 16 castrated mature female rats were used to assay each original extract. The assays of the material obtained after re-extraction (authors' method) were done on 3 castrated mature female rats. All these animals were primed with crystalline ketohydroxy-estrin and are known to respond to a minimum quantity of .5 gamma (5 I.U.).

The dose of extract for each animal was divided into 3 equal injections and given at 9 A.M. and 5 P.M. of the first day and at 9 A.M. of the second day. Vaginal smears were taken 48, 56, and 72 hours after the first injection and stained with thionin and phenol.‡ We considered one Rat Unit as the minimum quantity of extract necessary to produce a positive smear which contains only cornified epithelial cells.

It is noticed from Table II that the method described gives a greater yield than do the methods of Koch or Kurzrok. It will also be noted that Laqueur's method gives a yield approximately the same as the authors' but the time of extraction for bioassay is considerably longer.

Summary. A simple method is described whereby estrogenic substances can be extracted from human urine for bioassay. This method consists of adding 50 cc concentrated HCl (35-37%) per liter of urine; boiling for one hour and then cooling; vigorously shaking on a mechanical shaker for one hour with an equal volume of ethyl ether (U.S.P.); breaking up emulsion with 30-50 cc of sodium taurocholate (10% sol.) and discarding aqueous fraction; adding 6 cc sesame oil to the etherial extract and then evaporating ether on a hot water bath. This method is comparatively accurate and can be accomplished in about 3½ hours, thus making it an excellent routine method in an endocrine laboratory to expedite clinical and experimental work.

TABLE II.

Method	Hours Required for Entire Extraction	Yield/Liter R.U.	Yield/Liter I.U.
Laqueur	12	37.5	187
Koch	6	18	90
Kurzrok	53+	13+	66+
Authors'	3+	40	200

The methods were carried out on pooled specimen No. 1.

‡ To 100 cc of a 1% thionin solution was added 1 cc of phenol c.p. This solution was filtered before using.

10539

Inducing Land Stage of *Triturus viridescens* to Assume Water Habitat by Pituitary Implantations.

E. E. Reinke and C. S. Chadwick. (Introduced by J. M. Wolfe.)

From the Highlands Biological Laboratory, Highlands, N. C., and the Department of Biology, Vanderbilt University, Nashville.

The studies of Gage[1] and Pope[2] have shown the life cycle of *Triturus viridescens* to consist of 3 phases. (1) an aquatic larval stage of about 3 months' duration; (2) an immature, red-colored land stage, lasting from 2½ to 4 years and, finally, (3) an aquatic adult stage. While there are some variations in this typical life cycle, apparently depending upon the locality,[3, 4] the newt in the Blue Ridge Mountains of extreme Western North Carolina shows exactly the life cycle described by Gage and Pope, with a possible extension of the second stage into a fifth year.

The life cycle of Triturus is very interesting because of the 2 complete changes in habitat that take place. First, at 3 to 6 months of age, the metamorphosis from the aquatic larval to the terrestrial condition with the accompanying migration to land, and second, in the fourth to fifth year, the migration back to water as the animal approaches or reaches sexual maturity with consequent readaptation to a water habitat.

Metamorphosis among the Amphibia has been shown to be essentially an effect of the endocrine glands, particularly of the thyroid and indirectly the pituitary.[5] In our investigations on Triturus we have been interested, not in particular morphological changes, but in the relationship that might possibly exist between the endocrine glands and the phenomenon of *migration* from land to water and *vice versa*. Do the pituitary and thyroid glands, in addition to bringing about certain morphological changes, provide the "drive" which induces the change in habitat following metamorphosis? In seeking the answer to this question we sought first to determine the relation of the pituitary, if any, to the return of the land stage of Triturus to water. Our method was to implant whole pituitaries from adult water-stage newts intramuscularly into immature land stages (red efts) of various sizes and ages. The implanted efts were then

1 Gage, S. H., *Am. Nat.*, 1891, **25**, 1084.
2 Pope, P. H., *Ann. Carnegie Mus.*, 1924, **15**, 305.
3 Noble, G. K., *Am. Mus. Novitates*, 1926, No. 228.
4 Noble, G. K., *Am. Mus. Novitates*, 1929, No. 348.
5 Allen, B. M., *Biol. Rev.*, 1938, **13**, 1.

placed in observation containers of such a nature that either a land or a water habitat could be elected. Controls, either unimplanted or implanted with bits of tongue, liver or brain tissue, were placed in the containers along with the experimental animals and all were observed over a period of 6 weeks.

Thirty-two red efts of sizes ranging from 46 to 90 mm in overall length and of ages ranging from 1 to 4 years were successfully implanted. The dosage varied from 1 to 5 adult pituitaries for each eft and was given over a period of 2 to 6 days. A slightly greater number of male than female pituitaries were used for implantation purposes. Of the 32 animals implanted, regardless of their size or age, 31 voluntarily assumed a water habitat in from 2 to 6 days (46 to 144 hours). Only one animal, 77 mm in length, failed to enter water and in this case autopsy revealed faulty implantation.

Usually on the third to fourth day following the initial implant, the efts were observed in the process of shedding their skins. These molts in most cases occurred before the animals went to water. After about a week in water the experimental animals began to darken perceptibly dorsally and become lighter ventrally. As time passed the color tended more and more towards that of the adult. In certain animals that were observed as long as 6 weeks, the formation of a tail keel was noted. In all cases the skin assumed the smooth and moist condition of the adult.

None of the control animals entered water in spite of repeated, forcible attempts to make them do so. To test the completeness of their adaptation to a land habitat a number of normal efts of differing age and size were placed in a deep battery jar of pond water in such a way that they could bring their external nares to the surface but could not crawl from the water. Not only did these animals constantly struggle to get out but the majority of them died within 1 to 7 days. The few survivors when allowed the choice of remaining in water or going to land, immediately returned to land.

To determine the particular lobe of the pituitary in which the water drive factor is produced, 6 efts were implanted with anterior lobes and 6 others with posterior lobes. The 2 lobes were carefully separated by dissection after the whole gland had been removed from the donor. The 6 anterior lobe-implanted efts showed exactly the same sequence of changes as did those implanted with whole pituitaries. The posterior lobe-implanted animals behaved exactly as unimplanted efts.

Microscopic study of the thyroids and gonads of the pituitary-

implanted efts revealed that the thyroids had been stimulated, but there were no changes in the gonads as compared with the controls. The microscopic changes in the skin, particularly in the lateral line organs, were definitely towards the adult condition.

Although Adams[6] and Dawson[7] were able to induce efts to acquire the skin color and texture of the adult, the former by injecting phyone (an anterior lobe extract), the latter by intraperitoneal implants of frog anterior pituitaries, neither mention the assumption of a water habitat following such treatment.

Since the thyroids are stimulated by pituitary implants and since the thyroids have been shown to be necessary for normal metamorphosis among the Amphibia, we are conducting experiments at present to determine whether the effect of the pituitary in inducing the water drive is direct or through the intermediation of the thyroids. Though the experiments are by no means complete, pituitary implants have induced the water drive in 8 out of 8 completely thyroidectomized efts. We are testing also the effects of pituitary implants in completely gonadectomized animals.

These experiments demonstrate that the pituitary gland of adult *Triturus viridescens* produces a substance which, when properly administered, will induce the water drive in the red land stage of this species. The threshold dosage of pituitary substance necessary to bring about the water drive is low and the effect is reasonably quickly produced. The sequence of changes following implantation are, first, molting with the consequent assumption of a smooth, moist skin, second, the assumption of a water habitat and finally, a color change towards the adult condition.

---

[6] Adams, A. Elizabeth, *Anat. Rec.*, 1932, **52** (Supp.), 46.

[7] Dawson, A. B., *J. Exp. Zool.*, 1936, **74**, 221.

10540 P

Comparative Metabolism of Phosphorus in Normal and Lymphomatous Animals.*

JOHN H. LAWRENCE AND K. G. SCOTT.

From the Department of Internal Medicine, University of California Medical School, and the Crocker Radiation Laboratory, University of California.

When radioactive phosphorus is fed to normal rats, mice or chickens[1–4] in an ordinary diet, a large percentage of the phosphorus is deposited in the bony skeleton. This more or less selective deposition of phosphorus in bone, suggested to us the possibility of using radioactive phosphorus as a source of therapeutic irradiation in leukemia. In preliminary experiments, mice with lymphatic and myelogenous leukemia were fed radiophosphorus in relatively small amounts and there was no definite influence on the course of the disease. However, at autopsy, some of the tissues were assayed for activity and it was unexpectedly found that in leukemia there is an abnormality in the handling of a single dose of tagged phosphorus. Before pursuing this question further, it seemed to us important to determine first the total phosphorus content of leukemic, lymphomatous and normal tissues as a basis for further study. The findings[5] show that per gram wet weight, lymphomatous tissue or tissues infiltrated with lymphoid cells have no greater total phosphorus content than their normal analogues, *i. e.,* lymph glands and various uninfiltrated viscera. We wish here to report observations on normal and lymphomatous mice which show that a single dose of phosphorus is handled differently in the latter group of animals.

The phosphorus used in these experiments was made radioactive in the cyclotron of Lawrence and Cooksey[6] by bombarding red phos-

* This work was made possible only through the continued support to the Radiation Laboratory by the Chemical Foundation, the Research Corporation, and the Josiah Macy, Jr., Foundation. We wish also gratefully to acknowledge the cooperation of the entire staff of the Radiation Laboratory in the production of the radiophosphorus used in these experiments. Acknowledgment is also made for laboratory assistance furnished by the WPA.

1 Chiewitz, O., and Hevesy, G., *Nature,* 1935, **136**, 754.

2 Cook, S. F., and Scott, K. G., *Proc. Nat. Acad. Sci.,* 1937, **23**, 528.

3 Chiewitz, O., and Hevesy, G., *Det. Kgl. Danske Videnskabernes Selskab. Biologiske Meddelelser,* 1937, **13**, 9.

4 (a) Lawrence, J. H., *Handbook of Physical Therapy,* Amer. Med. Assn., 1938; (b) Hamilton, J. G., and Alles, Gordon A., *Am. J. Physiol.,* in press.

5 Tuttle, L. W., Scott, K. G., and Lawrence, J. H., to be published.

6 Lawrence, E. O., and Cooksey, D., *Phys. Rev.,* 1936, **50**, 1131.

phorus with deuterons having an energy of approximately eight million volts. As stated above, P^{32}, the radioactive isotope, is chemically similar to P^{31}, the naturally occurring element. It has a half life of about 14.8 days. Because of the beta ray emission, radiophosphorus can be detected and quantitated by means of an electroscope or a Geiger counter. After being made radioactive, the phosphorus was converted into a neutral solution of sodium phosphate and given to the animals subcutaneously in small "tracer" doses.

Forty Strong A strain mice,[7] approximately 3 months of age, and of about the same weight, were used in this experiment. Twenty of them were inoculated subcutaneously in one axilla with a transmissible lymphoma.[8] When large palpable tumors had developed, 15 days after inoculation, 5 tumor animals and 5 control animals were each given subcutaneously 0.5 cc of a sodium phosphate solution containing 20 microcuries of P^{32} and 1.57 mg Na_2HPO_4 per cc. Isotonicity was attained by adding NaCl. Two days later a similar group of 10 animals were given the same dose and 2 other groups of 10 on the 2 succeeding days. Twenty-four hours after the phosphorus was administered to the last group, all of the animals were sacrificed, the wet tissues of each mouse were weighed and prepared individually for activity analyses. Thus there was a group of 5 normal and 5 lymphomatous animals sacrificed 1, 2, 3 and 5 days after phosphorus administration.

The results are summarized in the figures. The average activities per gram wet weight of whole animal and various tissues are plotted against days after injection. It is to be noted that on the average, the phosphorus exchange per gram tissue of the mouse as a whole is about the same for normal and lymphomatous animals. However, it is noted that when individual tissues are examined there are differences. The percentages for muscle and total animal were about the same in both groups of animals. This is not true of bone in the lymphomatous animals, where the values are lower than normal, nor of liver where the 24-hour peak is lacking. Apparently, the deposition of radiophosphorus in tumor tissue occurred partially at the expense of bone and liver. Also lymphomatous tissue has a greater exchange than lymph gland, which might be considered its analogue. It is clear that the total phosphorus content of a tissue does not necessarily determine the uptake or exchange of a given dose of phosphorus in that tissue. The latter is determined by the rate of metabolism of the element in question in a particular tissue, and this may

7 Strong, L. C., *J. Heredity*, 1936, **27**, 21.

8 Lawrence, J. H., and Gardner, W. U., *Am. J. Cancer*, 1938, **33**, 112.

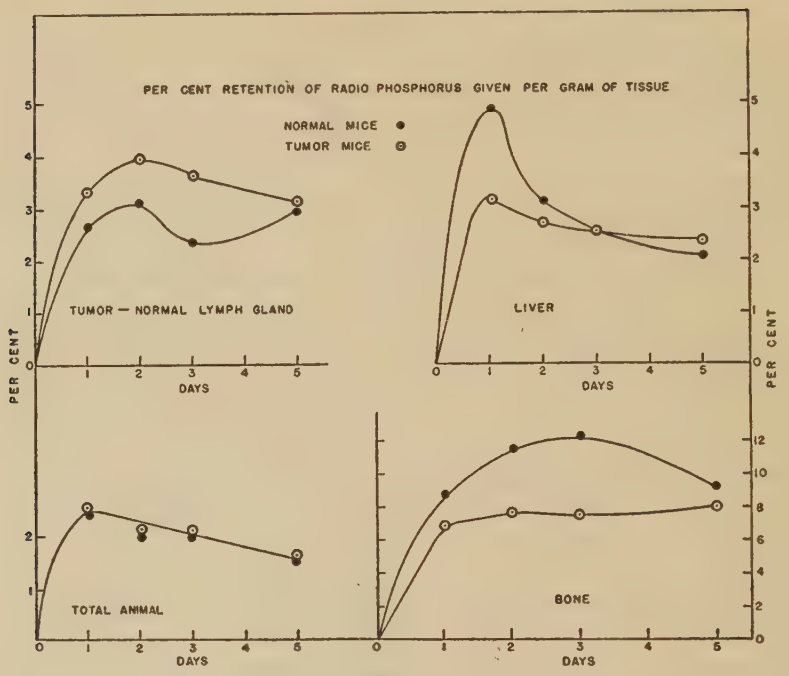


FIG. 1.

be complicated by the "laying down" of new tissue as is the case here.

The higher activities in bone and tumor tissue suggest the use of radiophosphorus as a source of therapeutic irradiation in conditions involving primarily the bone marrow. Recently by this method we have been able to produce remissions in cases of chronic leukemia (human), similar to those following the use of X-rays.

Further work is now in progress which includes the determination of the "tagged" phosphorus in the various organic and inorganic fractions and over longer periods of time, in an attempt to elucidate the variation in the handling of phosphorus by lymphomatous mice.

10541

Skin Impedance Findings in Mental Disease.

A. Barnett. (Introduced by W. M. Sperry.)

From the Department of Research Psychiatry, New York State Psychiatric Institute and Hospital.

It has been shown[1] that the electrical impedance per unit area of the skin may deviate from normal values not only in thyroid and other endocrine conditions but also in disturbances involving the autonomic and central nervous systems. Gross changes in skin capacity have been reported in certain types of mental disease.[2] It appeared to be of interest, therefore, to study a group of mental patients with a view to determining whether or not changes in the impedance properties of the skin could be found.

Impedance determinations were made by the 3-electrode method at 11,160 c.p.s., using a concentric and a disc electrode applied over the region of the biceps and triceps as described elsewhere.[1, 3]

The patients were not selected in any way and included all of the females (54) in the adult wards at the N. Y. State Psychiatric Institute. This group was composed predominantly of schizophrenics and psychoneurotics. The average age was 29 years.

A histogram of the results obtained is shown in Fig. 1 (dotted lines) and represents the distribution of the mean impedances of two 6 cm^2 skin areas measured on the anterior and posterior sides of the upper right arm. A corresponding histogram of the results obtained on 102 normal females in a previous investigation[1] (solid lines) is given for purposes of comparison.

As will be seen from Fig. 1, there is a considerable shift of the mentally diseased group toward low values, their mean impedance m falling well to the left of the normal mean M. The group of patients having impedances lying entirely outside the normal histogram on the low side are of particular interest since it is in this low impedance region that the values for thyrotoxics fall.[1] None of the patients in this low impedance group showed signs of thyrotoxicosis.

Since the tendency towards low impedance values in these patients might be due to a diminution in their skin phase angles, measurements were also made of the phase angle of the skin at 15,300 c.p.s. using the same electrodes. The phase angles, in every case, fell within

1 Barnett, A., *West. J. Surg.*, 1937, **45**, 540.

2 Roggenbau, C., and Lueg, W., *Monat. f. Psychiat. u. Neur.*, 1929, **73**, 301.

3 Barnett, A., *J. Physiol.*, 1938, **93**, 349.

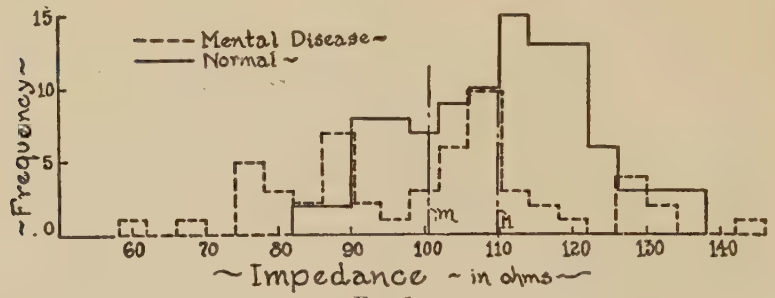


FIG. 1.

Histograms of skin impedance findings in normals (solid lines) and in the mentally diseased (dotted lines). Abscissæ in ohms, ordinates in frequency. The impedances represent values for a 6 cm^2 skin area.

normal limits and were well distributed over the normal range.[3] The skin phase angle of thyrotoxics has also been found to fall within normal limits.[1]

The fact that the skin phase angles of normal, thyrotoxic and mentally diseased subjects fall within the same limits while the impedances of thyrotoxics and mental patients show a group shift towards low values is of considerable interest. It may be interpreted to mean that although the quality of the dielectric (dielectric loss, power factor) remains the same, its thickness changes. This would be true for sheets of glass differing only in thickness. Their phase angles would be the same. The impedances would vary with the sheet thickness. It has been pointed out elsewhere[3] that impedances measured by the 3-electrode technic represent the properties of only the poorly conducting portions of the skin, *i. e.*, the epidermal layers. We appear to be dealing, then, with differences or changes in the thickness of the epidermis. This structure is known to be in a continuous process of growth. The thinning of the skin in thyrotoxicosis and its thickening in myxedema has long been known to clinicians. The effect of thyroid substance on tadpoles has been shown to be one of inhibiting growth and hastening differentiation,[4] *i. e.*, rapid evolution from tadpole to frog with symmetrical dwarfing. The thinning of the skin in thyrotoxicosis may, therefore, be due to a similar hastening of differentiation in the basal proliferating layers of the epidermis with "dwarfing" of the fully developed structure. In the case of mental patients, no such mechanism can be invoked for thinning since thyroid function appears to be, generally, normal. Evans[5] has shown that the proportion of growth hormone in the pituitary glands of adult cattle is as great as in those of young calves.

4 Gudernatsch, F., *Cold Spring Harbor Symp.*, 1934, **2**, 94.

5 Evans, H. M., *J. A. M. A.*, 1935, **104**, 464.

No satisfactory explanation for this interesting finding has, as yet, been given. The tendency towards low impedance skin values in mental disease may be a symptom of deficiency in growth hormone affecting directly or indirectly both the epidermis and the central nervous system. It is proposed, therefore, to study the therapeutic effect of growth hormone and other growth stimulating substances[6] in mental patients particularly where low skin impedance readings are obtained.

10542 P

Preparation of Follicle-Stimulating Extracts by the Use of Trypsin.*

W. H. MCSHAN AND ROLAND K. MEYER.

From the Department of Zoology, University of Wisconsin.

In a previous publication we have reported that the luteinizing activity in sheep pituitary gonadotropic extracts is destroyed by trypsin.[1] This has been confirmed by Chen and Van Dyke.[2] Our procedure for destroying the luteinizing activity has been utilized, therefore, in developing a convenient method for obtaining follicle-stimulating preparations.

The method of preparation is as follows: Acetone-dried sheep pituitary powder (100 g) was shaken with 1 liter of water and 0.5 cc of toluene for 12 hours and centrifuged. The extraction was repeated twice. The activity was recovered from the supernatant liquids by precipitation with acetone and centrifugation, after which the precipitate was suspended in 400 cc water, shaken and supercentrifuged.

The supercentrifuged supernatant liquid was treated at 37°C for 3.5 hours at pH 8 with 40 mg of trypsin† per gram of original pituitary powder and centrifuged. The precipitate was discarded. The clear supernatant liquid was placed in 50 cc centrifuge tubes and

6 Various authors in the Symposium on Growth, *Cold Spring Harbor Symp.*, 1934, **2**.

* Supported in part by a grant from the Wisconsin Alumni Research Foundation and the University of Wisconsin WPA Natural Science Research Program.

1 McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1938, **126**, 361.

2 Chen, G., and Van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 172.

† The trypsin used was samples No. 360427 and No. 390120 prepared by Fairchild Bros. and Foster, New York.

heated at 75°C for 20 minutes or until a precipitate formed which was eliminated by centrifugation. This step served to free the supernatant liquid of trypsin as determined by the method of Anson and Mirsky,[3] but with little if any loss in follicle-stimulating activity. A third inactive precipitate was formed by placing the heated supernatant liquid in cellophane tubes and dialyzing against 0.05 M sodium acetate buffer of pH 4. This precipitate was removed by centrifugation and the pH of the aqueous follicle-stimulating extract was adjusted to neutrality by dialysis against 0.05 N phosphate buffer. Certain of the extracts were used in this form for animal experimentation and others were sterilized by Seitz filtration for clinical use. There is apparently no significant change in the activity of the preparations after Seitz filtration (Table I), and no local or systemic reactions occurred when they were injected subcutaneously into human beings.

The nitrogen content of the follicle-stimulating preparations was 0.048% as determined by the micro-Kjeldahl method, and they gave a pink biuret test. The protein content of the aqueous preparations averaged 0.3% when calculated from the nitrogen content, or 6 mg per 0.5 g of original pituitary powder. The relation of the total nitrogen to the carbohydrate content of these preparations is given in another report.[4]

The data given in Table I show that when extracts prepared by the method just described were injected into normal 21-day-old female rats twice daily for 4.5 days, ovaries were obtained which

TABLE I.
Assay of Follicle-stimulating Preparations Made by Digestion of Sheep Pituitary Extract with Trypsin.

No. of Extract	Dose Trypsin, mg	No. of Rats†	Ovarian Response	
			Wt, mg	Qualitative
100WS	—	10	115	Many corpora lutea
FSH10WS	20	4	41	All follicles
FSH100WS	"	3	44	" "
FSH100WS*	"	3	39	" "
FSH50CS	"	3	35	" "
FSH50CS*	"	3	51	" "
FSH101WS	"	3	40	" "
FSH102WS	"	3	60	" "
FSH103WS	"	3	48	" "
FSH103WS*	"	3	43	" "

*After Seitz filtration.
† Each rat received 500 mg equivalent of dry pituitary powder.

3 Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1933-34, **17**, 151.

4 McShan, W. H., and Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 701.

contained only follicles. The uteri of these rats were distended with fluid in most cases while the vaginæ of approximately two-thirds of the rats were not open. The supercentrifuged aqueous extract before digestion with trypsin, however, produced ovaries which contained many corpora lutea together with undistended uteri and open vaginæ in all rats. These follicle-stimulating preparations were found to be free also of lactogenic and thyrotropic activities as indicated by the pigeon and young chick tests respectively.

Summary. A convenient method for the preparation of follicle-stimulating extracts from sheep pituitary powder by use of trypsin is described briefly. These preparations produced follicles only, when tested on normal female rats for 4.5 days, and were free of lactogenic and thyrotropic activities.

10543 P

Carbohydrate Properties of Pituitary Follicle-Stimulating and Luteinizing Preparations.*

W. H. McSHAN AND ROLAND K. MEYER.

From the Department of Zoology, University of Wisconsin.

We reported in a previous publication that the follicle-stimulating activity of sheep pituitary extract is destroyed by certain amylolytic enzymes, and on the basis of this evidence we suggested that the follicle-stimulating activity might be due to, or dependent upon, a carbohydrate grouping.[1] Such a point of view logically led to an examination of the carbohydrate properties of our follicle-stimulating and luteinizing hormone preparations. Anticipation of a part of our findings by Evans, *et al.*,[2] prompts us to publish a brief report of certain of our results at this time.

Follicle-stimulating preparations made by the trypsin method[3] and luteinizing extracts which were strong in thyrotropic activity were hydrolyzed with hydrochloric acid and the total reducing action of

* Supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1938, **126**, 361.

2 Evans, H. M., Frankel-Conrat, H., Simpson, M. E., and Li, C. H., *Science*, 1939, **89**, 249.

3 McShan, W. H., and Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 699.

the hydrolysates determined. In order to show the striking difference between the follicle-stimulating and luteinizing preparations the reducing action of the hydrolysates is expressed in terms of glucose which, however, does not infer that the carbohydrate involved in these extracts is glucose. The hydrolysates of the follicle-stimulating preparations gave reducing action equivalent to 1.22 mg of glucose per 0.5 g equivalent of pituitary powder as compared to 0.38 mg equivalent of glucose for the hydrolysates of the luteinizing extracts. The total reducing action expressed as glucose per 0.5 g equivalent of the dialyzed follicle-stimulating extracts was 20.3% of the total protein content of these extracts, which was 6 mg of protein per 0.5 g equivalent of pituitary powder based on Kjeldahl nitrogen determinations.[3] The value of 20.3% for our follicle-stimulating extracts was decreased, however, to 18.7% when the hydrolysates were treated with zinc hydroxide before the reducing action was determined.[4] This decrease with the zinc hydroxide treatment suggests that the value of 20.3% for the total reducing action was due in part to other reducing substances than carbohydrate. This leads to the belief that the value of 18.7% expressed as glucose is more nearly correct for our follicle-stimulating extracts. Furthermore, the basis on which the carbohydrate values were determined and calculated may account for the remaining difference between our value of 18.7% and that of 13% given by Evans, *et al.*,[2] for their follicle-stimulating extract.

The follicle-stimulating preparations used in the above reduction experiments gave a strong Molisch test for carbohydrate while the luteinizing extracts gave a weak test, which is in agreement with the difference in the reducing values for these respective preparations. The carbohydrate contained in the follicle-stimulating preparations became dialyzable on completion of the hydrolysis.

Another experiment was done in which the follicle-stimulating preparations were inactivated by electrodialysis while the luteinizing extracts were not inactivated. During the treatment of the follicle-stimulating extracts a precipitate formed which contained a small amount of carbohydrate while the most of the carbohydrate remained in the soluble fraction. The inactivation of these follicle-stimulating extracts on electrodialysis, may involve, therefore, a partial disruption of the carbohydrate grouping.

Further study was made of the inactivation of the follicle-stimulating activity by digestion of the extract with dialyzed ptyalin.[1] The digest was dialyzed after which the dialysate was concentrated. The total reducing action was determined on the concentrated dialysate and

[4] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

calculated in terms of glucose, which was equivalent to 0.056 mg per 0.5 g equivalent of the follicle-stimulating extract. The concentrated dialysate gave a positive Molisch test.

The results from the enzyme experiment described in which a part of the carbohydrate of the follicle-stimulating extracts became dialyzable on inactivation with ptyalin and the experiment on electrodialysis in which there was inactivation with a partial separation of the carbohydrate, do not prove, but further substantiate our previous suggestion that the follicle-stimulating activity may be associated with, or dependent upon a carbohydrate grouping.

Summary. The carbohydrate content of our follicle-stimulating and luteinizing preparations is given in terms of glucose calculated from the reducing action of the hydrolysates. Results from enzymatic inactivation and electrodialysis are given which suggest that the follicle-stimulating activity may be associated with a carbohydrate grouping.

10544

Protective Action of Sulfapyridine in Rabbits Infected with Pneumococci.*

W. P. LARSON, RAYMOND N. BIETER AND MILTON LEVINE.

From the Departments of Bacteriology and Pharmacology, University of Minnesota.

Most of the studies on the protective action of sulfanilamide and sulfapyridine on streptococcic and pneumococcic infections have been made on white mice. In the work here reported rabbits were used as the experimental animals. Intracutaneous inoculations were made in order to permit the observation of differences in the local lesions occurring in the treated and the control groups.

Rabbits weighing approximately 2 kilos were given 0.3 cc of a 1-100 dilution of an 8-hour culture of a Type II pneumococcus. The strain used had been transferred alternately through mice and veal broth for more than a year.

Intraperitoneal injections of 0.2 cc into mice, in dilutions of 1:10,000,000, kills 50% of the animals.

Sulfapyridine, 2-(sulfanilamido)-pyridine, was administered orally by suspending in 10% acacia and permitting the suspension to

* Aided by a grant from the research funds of the Graduate School. Merck & Co. kindly supplied the sulfapyridine used in these experiments.

TABLE I.

	No. inoculated	No. died	% mortality
Treated	25	6	24
Control	23	22	95.8

trickle down the throat from a large syringe. The treated animals were given 0.5 g one hour before inoculation, 0.5 g 3 hours after inoculation, and 0.5 g every 12 hours thereafter for a total of 5 g.

In addition to the difference in mortality, there was observed a marked difference in the lesions produced in the treated and control animals. Rhoads and Goodner[1] have reported oedema and a spread of the cutaneous lesion by gravity in rabbits inoculated endermally with pneumococci. These results were evident in our control group. The treated animals showed little or no oedema, and probably because of this, showed little or no spread by gravity. That an infection was present in the skin was evidenced by the area of inflammation in the skin of treated animals. The above authors have also stated that in occasional animals they find signs of hemorrhage in the skin. In the lesions of our control series there were extensive hemorrhages, caused either by the virulence of our strain or by the age of the culture. These started at the point of inoculation and spread to the periphery. In the center, they were discolored a bluish-black, but toward the edge showed the usual signs of fresh capillary damage. With 2 exceptions, the treated animals showed no signs of hemorrhage. These 2 showed a localized area of discoloration around the point of inoculation.

Control rabbits ate nothing up to the time of death, and consequently showed a progressive weight-loss. The temperature of these animals was from 2 to 3 degrees above the average normal temperature up to the time of death, at which time there was a marked rise in some, and a sharp fall to below normal in others. The treated animals showed the same high temperatures as the controls from 7 to 10 days after inoculation, at which time the temperature fell slowly to normal, and the rabbits resumed their normal diet after abstaining from food during the entire period of fever.

The recovered animals, when reinoculated with the type-specific pneumococcus, developed no signs of infection. A high degree of species-immunity was likewise developed against some of the pneumococcic types studied. Further studies on species-specific immunity are now in progress.

[1] Rhoads, C. P., and Goodner, K., *J. Exp. Med.*, 1931, **54**, 41.

10545 P

Experimental Production of Congestive Splenomegaly.

Louis M. Rousselot and William P. Thompson. (Introduced by M. N. Richter.)

From the Departments of Surgery and Medicine, and the Spleen Clinic, Presbyterian Hospital, New York City.

A variety of clinical studies on patients presenting the Banti syndrome have yielded much evidence to support the view that this syndrome is a secondary manifestation of a number of primary disturbances, resulting in chronic splenic vein hypertension.[1-6] These primary mechanisms, we believe, are obstructive in character within the portal bed, and may be intrahepatic (cirrhosis, Schistosomiasis, etc.) or extrahepatic (thrombophlebitis, external pressure on the vein, malformations, cavernomatous transformation of the splenic vein, etc.).

Over a period of several years we have tried to produce this syndrome experimentally by splenic vein constriction. The results have been uniformly disappointing, as either complete venous occlusion develops with splenic atrophy, or an adequate collateral promptly forms with no alteration in the size of the spleen.

The similarity between lesions produced by Schistosoma ova and small particles of silica suggested that a perivascular fibrosis within the liver, with progressive intrahepatic portal vein constriction, might be produced by injecting silica directly into the portal vein.

Five adult and 3 young dogs have been used and similarly studied. All animals have had several injections of a sterile saline suspension of silicon dioxide directly into the splenic vein.* Each injection is made at the time of an exploratory operation. The individual silicious particles are 1-3 micra in diameter. At the time of writing, 3 animals have progressed to a late enough stage of cirrhosis to produce splenic vein hypertension with splenomegaly. The latter occurred only when enough silica is given, in our cases 6.0 g, and only when sufficient time has elapsed to produce an advanced liver cirrhosis.

1 Klemperer, R., *Arch. Path.*, 1928, **6**, 353.

2 Larrabee, R. C., *Am. J. M. Sci.*, 1934, **188**, 745.

3 Campbell, H. E., *Chinese M. J.*, 1936, **50**, 1561.

4 Rousselot, L. M., *J. A. M. A.*, 1936, **107**, 1788.

5 Rousselot, L. M., *Bull. N. Y. Ac. Med.*, 1939, **15**, 188.

6 Thompson, W. P., Caughey, Whipple, Rousselot, *J. Clin. Invest.*, 1937, **16**, 571.

* The saline suspensions of silica were prepared for us by Dr. Leroy U. Gardner, director of the Saranac Laboratories, Saranac, N. Y.

This takes approximately 24 months. Of the remaining 5 animals, one (703) was sacrificed to study the early lesions of the sequence; another (708) was autopsied after developing "congestive splenomegaly"; one (287) died of postoperative hemorrhage from the site of the venipuncture wound following silica injection; 2 animals (285, 286) have received 6.0 and 5.0 g respectively of silica, but insufficient time has elapsed to develop to the stage of congestive splenomegaly.

During the first 6 months, no gross pathological changes can be detected in liver or spleen, and only a few early silicotic nodules are seen microscopically in the liver biopsy. This failure of the silica to settle in greater concentration in the liver has been a matter of extreme interest to us. After autopsy examination of the first animal in the early state (5.9 g of silica injected), it was found that greatly enlarged, hard lymph nodes were present in the hepatic chain draining the liver. Histologically these nodes proved to be completely replaced by silicotic nodules. This lymphatic lesion has consistently appeared in all our animals prior to the hepatic retention of the silica. The migration of inert particulate matter from the portal vein into the lymphatic vessels surrounding the veins in the portal spaces and thence to issue from the liver via the efferent lymphatics has been noted.[7, 8, 9] Such was the route taken by the silica in our animals. Only after the efferent lymphatics have been blocked, and this occurs after approximately 3.0 g of silica has been injected, does the silica accumulate in the liver in sufficient amounts to produce a progressive cirrhosis. Toward the end of the 2-year period the liver is enlarged, hard, and fibrotic. With this a splenomegaly is apparent and an associated rich venous collateral has developed. The spleens in the 3 completed experiments have reached very large proportions. (See Table I.)

At the end of the experiment in these same 3 animals a splenic vein hypertension has likewise been present as compared with normal venous pressures in the saphenous vein. (See table.) The readings as recorded are 2-3 times higher in the splenic vein than in the peripheral vein under the same conditions.

Microscopic examination of the liver at the stage of splenomegaly shows typical silicotic nodules of varying ages, located mainly in the periportal areas and occasionally in the mid-portion of the lobule. In the spleen a fine fibrosis is apparent. A few silica particles are found

[7] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. Lond.*, 1906, **78**, 455.

[8] Kiernan, *Phil. Trans.*, 1833, 753, quoted by Herring.

[9] Schafer, *Proc. Roy. Soc. Edin.*, 1902, **24**, 65, quoted by Herring.

TABLE I.

Animal No.	Amount of silica, g	Duration followed, mo.	L—Living A—Autopsied	Liver	Spleen	Venous Pressure in mm of water	
708	6.0	26	A	Hard; cirrhotic	Large 19 x 5.5 x 3.5 cm	Splenic vein—295 Saphenous vein—63	
27	6.0	24	L	" "	Very large 31 x 7 x 3 cm	Splenic vein—230 Saphenous vein—35	
6	6.0	23	L	" "	Very large 25 x 8.5 x 3.5 cm	Splenic vein—260 Saphenous vein—60	
690	5.6	37	L	" "	Moderately enlarged	Splenic vein—225 Saphenous vein—140	Reading equivocal; technical difficulty
703	5.9	18	A	Normal size and consistency; early silicotic nodules	Not enlarged	—	
285	6.0	6	L	Firm; early cirrhosis	Moderately enlarged 16 x 5.5 x 1.5 cm	—	
286	5.0	7	L	Firm; early cirrhosis	Moderately enlarged	—	
287	6.0	8	A	Slightly enlarged; early cirrhosis	Very large 24 x 9.5 x 3.0 cm Weight 300 g	—	Size of spleen determined after death from hemorrhage

without any significant reaction. These particles occur in such small numbers that it is the opinion of Dr. Leroy Gardner and ourselves that they are insufficient to account for the marked enlargement of the spleen.

Summary. The injection of fine silicious particles directly into the splenic vein in the amounts described will produce a progressive cirrhosis of the liver. Secondary to this a state of splenic vein hypertension has been produced with a concomitant congestive splenomegaly.

10546 P

Reduction of Experimental Renal Hypertension by Pexis of Spleen or Omentum to the Kidney.

JAMES S. MANSFIELD, DAVID M. WEEKS,* ALFRED STEINER AND JOSEPH VICTOR.

From the Research Division of Chronic Diseases, Department of Hospitals, City of New York, and the Department of Medicine, College of Physicians and Surgeons, Columbia University.

The effect of the pexis between kidney and the spleen or omentum upon the blood pressure of dogs rendered hypertensive by Goldblatt's[1] method of renal constriction has been studied in this laboratory since 1936. Sixty-five dogs have been employed in the development of 2 suitable technics for establishing organ pexis to such ischemic kidneys. Seven animals survived the various surgical procedures for producing carotid loop,[2] hypertension[1] and the union of the organs.

Only 2 died as the result of the latter operation. Fifty-six dogs died or were discarded because of hemorrhage from the carotid loop employed for blood pressure determination, anesthetic deaths, uremia from infarction of the kidneys, distemper, and operative infection. Postoperative shock was insignificant following the pexis which was performed one to 3 months after the hypertension was established. Control operations in which the omentum and spleen were manipulated did not result in a significant prolonged fall in blood pressure.

The blood pressure of all of the 7 animals was lowered within 4 days following the union. Three of these, following the pexis of

* Instructor in Surgery, New York Medical College.

1 Goldblatt, Harry, *Experimental Hypertension Induced by Renal Ischemia*, The Harvey Lectures, 1937-38, Williams and Wilkins Co., Baltimore, 1938.

2 Van Leersum, E. C., *Arch. Ges. Physiol.*, 1911, **142**, 377.

TABLE I.
Effect of Pexis of Omentum or Spleen to Kidney on Blood Pressure of Hypertensive Dogs.

Type of Pexis and Dog No.	Blood Pressure, mm/Hg			Duration of Fall, weeks
	Initial	Following Constriction of Renal Arteries	Following Pexis	
Spleen-Kidney				
72	150	250-255	180	23*
75	145-150	230-245	135-190	6*
95	160-200	220-285	165-185	8†
116	130-150	220-250	155-175	5†
Omentum-Kidney				
37	155-140	240-280	175-185	5‡
41	165-170	230-250	165-180	4‡
74	170	220-240	170-180	1‡

*Sacrificed.
† Still living.
‡Blood pressure subsequently reached hypertensive level.

decapsulated renal surface with omentum, had temporary reversal of hypertension lasting 6-38 days. (Table I.) The other 4 animals in which the longitudinal cut surface of the spleen was attached to the renal surface or imbedded (Animals Nos. 95 and 116) in an incision through the long axis of the kidney down to the pelvis, showed a continuous decline in blood pressure toward normal, persisting 5 weeks to 6 months. (Table I.)

To verify the existence of a collateral circulation, India ink was injected into the splenic artery of an excised preparation consisting of an adherent kidney and spleen. Both renal arteries had been constricted for 2 months and the spleen joined to the kidney for one month. Ink particles were visible in the capillaries about the convoluted tubules but not in those of the glomeruli. In 2 other dogs, Nos. 72 and 74, there was direct communication between the capillaries of the renal parenchyma and the sinusoids in the connective tissue of the adhesions.

These data suggest that the hypertension produced by constriction of the renal arteries is lowered by pexis between the kidney and the omentum or the spleen. The reduction in blood pressure with the omental union was only temporary whereas the reduction with the splenic pexis was sustained. This difference may reflect the degree of effective collateral circulation.

10547 P

Determination of Threonine.

RICHARD J. BLOCK AND DIANA BOLLING.

From the Department of Chemistry, New York State Psychiatric Institute and Hospital.

Criegee, Kraft and Rank[1] showed that glycols and related compounds are split by lead tetraäcetate to yield ketones or aldehydes. Threonine ($CH_3CHOHCHNH_2COOH$) should, therefore, yield acetaldehyde when oxidized by lead tetraäcetate. Eegriwe[2] described a highly sensitive color reaction for the qualitative estimation of lactic acid based upon the oxidation of the acid with hot concentrated sulfuric acid to acetaldehyde; from the acetaldehyde an intense violet color is produced by condensation with p-hydroxydiphenyl. Miller and Muntz[3] modified Eegriwe's method and they have shown that the color is not given by a number of other acids such as glycolic, oxalic, formic, 2,3-dihydroxybutyric, etc. The apparent specificity of the p-hydroxydiphenyl test for acetaldehyde suggested that it would be of value in the quantitative estimation of threonine.

The following method can be used for the determination of threonine alone or in the presence of other amino-acids: 25 cc of glacial acetic acid containing from 0.5 to 1.5 mg of threonine and 1 g of lead tetraäcetate are kept at 30° for one hour. The acetaldehyde is removed from the reaction mixture during the course of the oxidation by moderately rapid aeration and taken up in 10 cc of concentrated sulfuric acid containing 5 drops of water and 100 mg of p-hydroxydiphenyl in suspension. At the end of the hour, the excess p-hydroxydiphenyl is dissolved by warming to 100°. The solution may be diluted to volume with concentrated H_2SO_4. The amount of color is in direct proportion to the quantity of threonine used. Control tests with alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, norleucine, phenylalanine, proline, serine, tryptophane, tyrosine, valine and mixtures of the same were negative. Hydroxyglutamic acid was not available for test. Preliminary experiments suggest the applicability of these reactions to the determination of threonine in proteins and other biological material.

1 Criegee, R., Kraft, L., and Rank, B., *Lieb. Ann.*, 1933, **507**, 159.

2 Eegriwe, E., *Z. anal. Chem.*, 1933, **95**, 323.

3 Miller, B. F., and Muntz, J. A., *J. Biol. Chem.*, 1938, **126**, 413.